RELATIONSHIP BETWEEN NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) METABOLISM AND INOSITOL BIOSYNTHESIS

A Dissertation

Presented to the Faulty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirement for the Degree of

Doctor of Philosophy

by

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August 2009

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Cornell University 2009

I found that the presence of the phospholipid precursor, inositol, in the growth medium alters NAD^+ levels, as well as, expression levels of genes involved in NAD^+ metabolism. NAD^+ levels increased in the absence of inositol compared to the levels in the presence of inositol.

My initial discovery of a relatively weak Ino^- phenotype at 37^oC associated with the *npt1*Δ mutant in the NAD^+ salvage pathway and the fact that this phenotype is partially suppressed by removal of nicotinic acid (NA) from the growth medium added further evidence of a connection between NAD^+ and inositol metabolism. Changes in the level of *INO1* expression and phospholipid composition in *npt1*Δ were restored to wild type levels when NA was removed from the growth medium. The fact that the Ino^- phenotype of the *npt1*Δ mutant was strongest when NA was present was surprising because the *npt1*Δ mutant is unable to use NA as a precursor for NAD^+ biosynthesis.

Consistent with the nature of the metabolic defect in the *npt1*Δ mutant, I subsequently found that the effect of NA on the Ino^- phenotype of *npt1*Δ was not correlated to changes in either intracellular NAD^+ or NA levels. Moreover, deletion of the gene encoding the sirutins, Hst1p, in the genetic background, *npt1*Δ, and/or addition of nicotinamide (NAM), an inhibitor of sirtuins, to the growth medium resulted in a stronger Ino^- phenotype. Together these results suggested that the Ino^- phenotype of *npt1*Δ might be related to changes in phospholipid homeostasis and *INO1* expression influenced by exogenous NA as well as, the activity of sirtuins, Sir2p,

Hst1p or Hst2p, especially at higher temperatures. Sirtuins are NAD^+-dependent protein deacetylases involved in the regulation of a wide ranged of cellular process.

Consistently low NAD^+ levels in the *npt1*Δ strain under all conditions tested may result in decreased activity of sirtuins. *BNA2* expression level in the *npt1*Δ strain was highly and transiently increased in the absence of inositol whether NA was present or not. A similar expression pattern was seen in the *hst1*Δ strain. Hst1p is known as a negative regulator of *BNA2* and other genes in *de novo* pathway for NAD^+ synthesis. Hst1p is believed to be recruited to the promoter of these genes where it deacetylates histones, resulting in localized gene silencing. Thus, increased *BNA2* expression levels in *npt1*Δ cells are likely to be due to decreased activity of Hst1p when NAD^+ levels are low. Therefore, when Hst1p is inactive, upregulation of *BNA2* occurs in response to increasing cellular demand for NAD^+. My results further suggest that NAD^+ levels or synthesis and/or turnover may be responding to increasing cellular stress, which occurs in the absence of inositol and at higher growth temperatures.

In this study, I examined the interaction of inositol biosynthesis and NAD^+ metabolism and the effects that these metabolites exert on regulation affecting each other. My data indicate that NAD^+ levels and sirtuin activity affect lipid homeostasis and cell viability under conditions of cellular stress created by high temperature and the absence of inositol. These results suggest that sirtuins and NAD^+ metabolism play crucial, but previously undescribable roles under conditions of cellular stress.

BIOGRAPHICAL SKETCH

Sojin Lee was born in Seoul, Korea on December 30, 1974. She earned her Bachelors degree from Seoul Women's University in February 1997 and her master degree from Seoul National University in February 1999, the title is "Transglycosylation of naringin by *Bacillus stearothermophilus* maltogenic amylase to give glycosylated naringin, *J. Agric & Food Chem*., 47(9), 3669-3674 (1999)."

After that, she worked in National Rural Living Science Institute and Green Tek21 Co., Ltd as a researcher. During working, she published the paper "Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from *Stevia rebaudiana* leaves, *Chromatographia*, 55(10), 617-620 (2002).

She joined in the graduate field of Food Science & Technology at Cornell in Fall 2002. Shte joined the laboratory of Susan Henry Lab in summer 2003. She studied about nicotinamide adenine dinucleotide (NAD^+) metabolism connecting phospholipid metabolism.

After receve her Ph.D. Degree from Cornell, she will stay more in Henry lab to publish her thesis.

To my God, with His mercy and grace I have been here

To my parents, Rimtaig Lee and Soonja Kim, for their sacrifies and encouragement, which were the powers to overcome

To my advisor, Dr. Susan A. Henry, without her endurance and patience I would not have finished my study

ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Susan A. Henry for teaching, supporting and encouraging me for six years. Her insight and advice aided the writing of this thesis in innumberable ways. She is the greatest person as a scientist, a professor and a friend, who I have met in my life. The relationship I have with her was through the blessing and mercy of God.

I would like to thank my committee members, Dr. Eric Alani, Dr. Barbar Baird and Dr. Randy Worobo for a lot of insightful comments, advice and hard questions, whenever we had meetings.

I would like to thank my parents, Rimtaig Lee and Soonja Kim, for their unconditional love and supporting me. And I would also like to show my appreciation to my family, Youngmi Lee, Heejin Lee and Yongkwun Lee for their continuous support in my study.

I would like to express the deepest appreciation to Henry lab members, Laura Gasper, Steve Jesch, Lilia Nunez, Mannel Aregullin, Manuel Villa, Yufang Chang, and Jessica Musa for their friendly help in experiments and preparing presentations.

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CHAPTER ONE
INTRODUCTION

A. Inositol biosynthesis in phospholipid metabolism

A.1 Inositol functions in biochemical, nutritional, and clinical aspects

Inositol is six-carbon sugar alcohol and exists in a number of stereoisomers. Among them, L-myo-inositol is an essential soluble precursor of phospholipid metabolism in eukaryotes. Phospholipids are major components of all eukaryotic biological membranes along with sphingolipids and sterols. Inositol-containing compounds such as phosphoinositides or inositol polyphosphates, play diverse biological roles such as signal transduction, second messenger signaling, stress response, and cell wall biogenesis (Exton 1986; Lee, Fisher et al. 1991; Majumder, Johnson et al. 1997; Nunez, Jesch et al. 2008). For example, inositol triphosphate (IP_3) hydrolyzed from phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) is a second messenger in mammal cells resulting in intracellular calcium mobilization (Berridge and Irvine 1989; Berridge 1994). Phytic acid, inositol hexakisphosphate, synthesized from inositol polyphosphates is present in high levels in staple foods like cereals, corn and rice. However, phytic acid causes problems in bioavailability and utilization of calcium, iron and zinc in mammals due to its activity as a chelating agent (Sandberg 1991). Inositol is also a critical nutrient in infant formulas which do not contain milk. It is reported that supplemental inositol results in increased survival of preterm infants with respiratory distress syndrome as well as decreased the incidence of bronchopulmonary and retinopathy of prematurity (Hallman, Bry et al. 1992).

Dietary inositol level influences the concentration of free inositol and inositol-containing phospholipids in mammalian tissues and cells. Inositol deficient diets have been implicated in the impaired released of plasma lipoprotein, increased fatty acid

mobilization from adipose tissues, enhanced fatty acid synthesis in liver and finally induction of triacylglycerol accumulation in hepatic cells (Holub 1986). Changed levels of inositol and other components of phospholipid metabolism have been implicated in various disease states including diabetes, renal disorders, manic depression and certain cancers (Holub 1986).

A.2. *De novo* inositol biosynthesis in yeast

In baker's yeast, *Saccharomyces cerevisiae*, as in other eukaryotes, inositol is synthesized *de novo* from glucose-6-phosphate (Glu-6-P). This pathway has been well characterized in yeast (Carman and Henry 1999). The first step of the pathway is the conversion of Glu-6-P to inositol-3-phosphate (Ino-3-P) by inositol-3-phosphate synthase (Ino1p) encoded by the *INO1* gene. Subsequently, Ino-3-P is dephosphorylated into inositol by inositol-monophosphatase (Inm1p) encoded by the *INM1* gene (figure 1). The synthesis of Ino-3-P by Ino1p is the rate-limiting step in this pathway (Donahue and Henry 1981). Ino1p requires nicotinamide adenine dinucleotide (NAD^+) as a cofactor for its activity (Maeda and Eisenberg 1980; Mauck, Wong et al. 1980; Byun and Jenness 1981). It was recently reported that Ino1p is a homotetramer and the monomer is divided into three major domains. These domains include a central domain, which contains the majority of contact zone between the monomers, a Rossmann-fold domain, which is a NAD^+-binding domain, and a third domain, which contains a β-sheet and the catalytic domain (Stein and Geiger 2002; Jin and Geiger 2003). The reaction catalyzed by Ino1p occurs in three successive steps; an oxidation, an intramolecular aldol cyclization and a reduction; all involving the same active site (Barnett and Corina 1968; Sherman, Stewart et al. 1969). Ino1p homologues are found in virtually all eukaryotic organisms whose genomes have been fully sequenced and the protein is highly conserved. In humans, the h*INO1* gene

contains the upstream stimulating factor (USF) element in its promoter. The core binding sequence (GYCARTGC) of the USF element is similar to the core binding sequence (CAYRTG) of the yeast UAS_{ino} element. There is 56% amino acid sequence similarity between hIno1p and yeast Ino1p (Shamir, Shaltiel et al. 2003). However, it has not been reported whether transcription of h*INO1*, like the yeast *INO1* gene, responds to inositol availability.

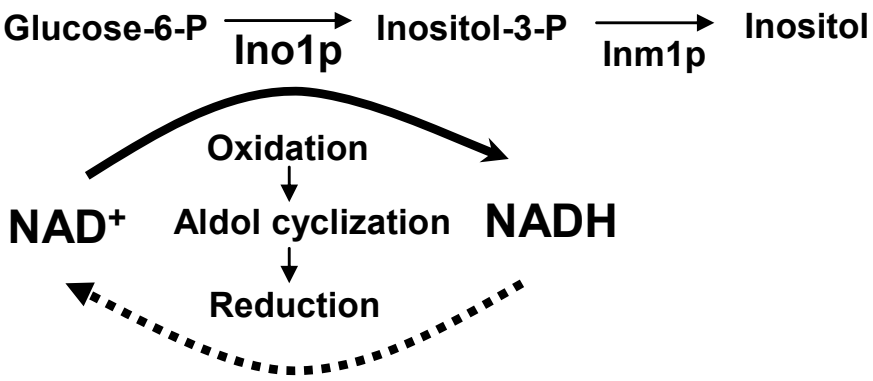


Figure 1. A. Inositol Biosynthesis and the Ino1p requirement for NAD^+ as a cofactor. Inositol-3-phosphate synthase (Ino1p) catalyzes the conversion of glucose-6-phosphate (Glucose-6-P) to inositol-3-phosphate (Inositol-3-P) through the three steps, oxidation, aldolcyclization and reduction. Ino-3-P is dephosphorylated into inositol by inositol-monophosphatase (Inm1p) (Barnett and Corina 1968; Sherman, Stewart et al. 1969; Byun and Jenness 1981). The redox reactions using NAD^+ and NADH are essential to Ino1p activity.

A. 3. Transcriptional regulation of *INO1* gene in phospholipid metabolism

In yeast, *INO1* transcription is highly regulated in response to inositol availability (Carman and Henry 1989; Carman and Henry 1999). *INO1* contains the

upstream activation element, UAS_{ino} (CATGTGAAAT) as well as the upstream repression element, URS1, in its promoter (Lopes and Henry 1991; Lopes, Hirsch et al. 1991; Lopes, Schulze et al. 1993). The Ino2p-Ino4p heterodimer binds to the UAS_{ino} elements activating transcription (Ambroziak and Henry 1994; Nikoloff and Henry 1994). Opi1p also interacts with the *INO1* promoter via an interaction with Ino2p to repress *INO1* transcription (Wagner, Dietz et al. 2001). Opi1p binds phosphatic acid (PA) and interacts with Scs2p, a transmembrane protein, in the endoplasmic reticulum (ER) in the absence of inositol (Loewen, Gaspar et al. 2004). When inositol is added in the medium, synthesis of phosphatidylinositol (PI) dramatically increases leading to immediate consumption of PA, a precursor of PI. Low PA levels induce translocation of Opi1p to the nucleus where it leads to repression of *INO1* (Loewen, Gaspar et al. 2004) (figure 1.B). In yeast, deletion of *OPI1* results in overproduction of inositol (Opi^- phenotype) due to high level constitutive expression of *INO1* (Greenberg, Reiner et al. 1982; Henry and Patton-Vogt 1998). Deletion of *INO1*, *INO2* or *INO4* results in inositol auxotrophy (Ino^- phenotype) (Klig and Henry 1984; Hirsch and Henry 1986; Greenberg and Lopes 1996).

INO1 expression levels are sensitive to growth stage as well as inositol availability. *INO1* expression peaks at mid-logarithmic phase in inositol deficient media, decreasing rapidly in late-logarithmic phase and is fully repressed at stationary phase (Lamping, Luckl et al. 1994; Jiranek, Graves et al. 1998). *INO1* transcription is also regulated in response to the availability of nutrients such as nitrogen and glucose (Patton-Vogt and Henry 1998). Recently, it was reported that transcription of *INO1* is affected by the activity of the proteins involved in inositol biosynthesis, such as Ino1p. For example, valporate, a drug used in treatment of bipolar disorder, inhibits Ino1p enzymatic activity which leads to a decrease in intracellular inositol levels. Wild type cells growing in synthetic medium containing valporate require inositol (i.e., valporate

induces a growth defect in wild type cells grown in medium lacking inositol), but *INO1* expression levels are actually increased under these conditions (Ju, Shaltiel et al. 2004). Lithium, another drug used to treat depression, causes inositol depletion by inhibition of Inm1p, which also induces increased *INO1* expression levels (Vaden, Ding et al. 2001). In addition, dihydroxyacetone phosphate (DHAP) and glyceraldehydes-3-phosphate, both precursors of PA (Figure 1.C), as well as oxaloacetate, are all reported to inhibit Ino1p activity (Shi, Vaden et al. 2005). When these compounds are added to the growth medium, wild type cells are dependent on inositol supplementation (i.e., they will not grow in medium lacking inositol (I-medium)) even though the *INO1* gene is transcribed.

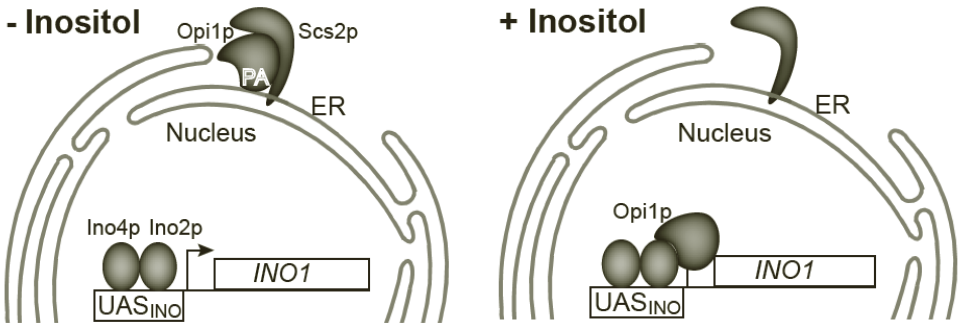


Figure 1.B. Regulation transcription of *INO1* by Ino2p-Ino4p and Opi1p. In the absence of inositol (-Inositol), Opi1p, a negative regulator of transcription of the *INO1* gene, is located at the ER through interaction with the transmembrane protein, Scs2p as well as by branching to PA. The Ino4p and Ino2p, heteromdimer, is continuously bound to UAS_{ino} to activate *INO1* gene. Addition of inositol (+Inositol) causes PA consumption resulting in Opi1p translocation to the nucleus. In the nucleus, Opi1p interacts with Ino2p, resulting repression of the *INO1* gene (Jesch, Zhao et al. 2005).

A. 4. Affect of inositol supplementation on the levels of phospholipid metabolites

Major phospholipids of the eukaryotic cell include phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Figure1.C). These phospholipids are derived from cytidine diphosphate-diacylglycerol (CDP-DAG) and diacylglycerol (DAG). CDP-DAG and DAG are synthesized from PA by phosphatide cytidyltransferase encoded by the *CDS1* gene and phosphatidate phosphatase encoded by the *PAH1* gene (Figure 1.C). PA is a precursor of all phospholipids and PA levels play an important role in regulation of phospholipid metabolites as described previously (Henry and Patton-Vogt 1998; Loewen, Gaspar et al. 2004; Carman and Henry 2007) (Figure 1.B).

Both endogenously synthesized and exogenously provided inositol combine with CDP-DAG to synthesize PI in a reaction catalyzed by phosphatidylinositol synthase, encoded by the *PIS1* gene. PI serves as a precursor of inositol-containing metabolites or compounds, such as sphingolipids (SpLip), phosphoinositides (PIPs), glycosylphosphatidylinositol and soluble inositol polyphosphates (IPs) (Figure 1.C). In yeast, inositol supplementation has been shown to affect levels of PI (Gaspar, Aregullin et al. 2006). In the presence of inositol, PI levels are increased whereas PS levels are decreased; leading to decreased PE and PC levels (Kelley, Bailis et al. 1988; Loewen, Gaspar et al. 2004; Gaspar, Aregullin et al. 2006). In contrast, in the absence of inositol, PS, PE and PC levels are increased and PI levels are decreased. The addition of exogenous inositol to cells grown in the absence of exogenous inositol induces dramatic changes in phospholipid metabolism. PI levels increase rapidly whereas CDP-DAG, PA and PC decrease (Gaspar, Aregullin et al. 2006). Also, in inositol deficient growth conditions, free fatty acid levels are elevated in wild type cells compared with the levels observed in cells grown in medium containing inositol (Gaspar, Aregullin et al. 2006).

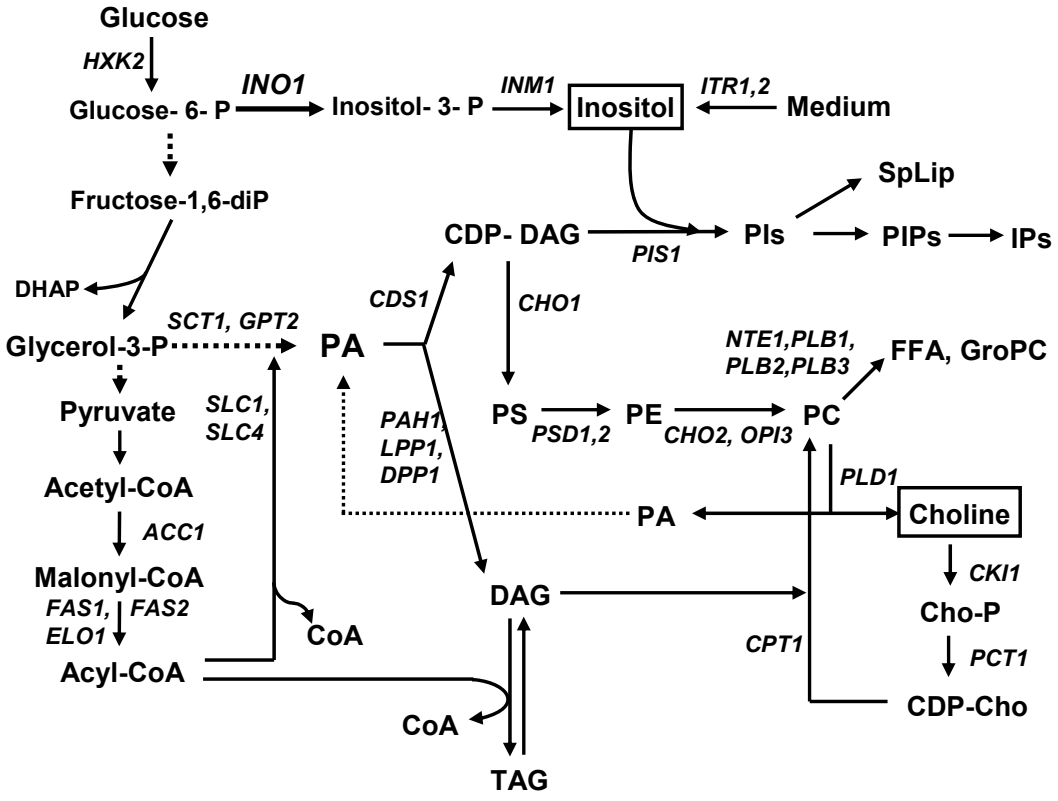


Figure 1.C. Pathway of phospholipid metabolism. Designations for the structural genes encoding enzymes catalyzing specific metabolic conversions are shown adjacent to arrows. Inositol and choline (in boxes) are major precursors of phospholipid metabolism. The solid arrows represent routes of metabolic conversion. Dashed arrows indicate conversions that require more than one enzymatic step. Glucose-3-P, glucose-3-phosphate; Inositol-3-P, inositol-3-phosphate; DHAP, Dihydroxyacetone phosphate; Fructose-1.6-diP, Fructose-1,6-diphosphate; Gly-3-P, glyceraldehyde-3-phosphate; CDP-DAG, cytidine diphosphate-diacylglycerol; PI, phosphatidylinositol; PIP, phosphoinositide; IP, inositol phosphates; SpLip, sphingolipids; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylehtanolamine; PA, phosphatidic acid; Cho, choline; Cho-P, Choline phosphate; CDP-Cho, cytidine diphosphate-choline; DAG, diacyl glycerol; TAG, triacylglycerol; GroPC, glycerophosphocholine.

B. Nicotinamide adenine dinucleotide (NAD^+) metabolism

B.1. Precursors of NAD^+ metabolism and their biological and nutritional effects

Nicotinic acid (NA), nicotinamide (NAM), nicotinamide ribose (NR) and tryptophan are precursors of NAD^+ in eukaryotic cells. Some prokaryotic cells utilize aspartic acid or glutamic acid as precursors instead of tryptophan (Begley, Kinsland et al. 2001). NA and NAM are collectively referred to as vitamin B_3, niacin. These precursors are derived from meats, legumes, seeds, milk, green leafy vegetables, fish and yeast. Tryptophan and niacin-poor diets result in pellagra, a nutritional deficiency disease (DiPalma and Thayer 1991). Specifically, it has been reported that corn-rich diets are associated with a high occurrence of pellagra (A Plague of Corn; The Social History of Pellagra, Daphne A. Roe, 1973). Dietary NA has been reported to be a lipid-reducing agent leading to a decrease in low density lipoprotein (LDL) cholesterol and triglycerides (TAG) and an increase in high density lipoprotein (HDL) cholesterol in plasma (Grundy, Mok et al. 1981; Ganji, Kamanna et al. 2003; Chapman, Assmann et al. 2004). NAD^+ is involved in many biological processes, including the production of energy, the synthesis of fatty acids, cholesterol and steroids, signal transduction, the regulation of gene expression and the maintenance of genomic integrity (Hageman and Stierum 2001). Dietary niacin reportedly affects the levels of NAD^+. For example, diets deficient in niacin were reported to result in an 80% reduction in NAD^+ levels in bone marrow (Boyonoski, Spronck et al. 2002).

B. 2. NAD^+ functions in cellular process

NAD^+ is an essential co-enzyme for redox reactions like glycolysis and respiration in mitochondria. NAD^+ is reduced to NADH (reduced form) and phosphorylated to $NADP^+$ which is reduced to NADPH. These derived forms play

important roles as reducing equivalents (NADH and NADPH) and as electron transporters (NAD^+ and $NADP^+$) in anabolic and catabolic pathways and energy metabolism. Intracellular NAD(H) exists free and bound forms (Blinova, Carroll et al. 2005). Also, NAD^+ is required for the activity of numerous proteins, such as sirtuins, poly (ADP-ribose) polymerase (PARP), mono-ADP ribosyltransferase (ART), as well as other proteins responsible for altering the assembly of the nucleoprotein complex involved in post-translational modification in eukaryotic cells (Michan and Sinclair 2007). Sirtuins, such as Sir2p in yeast and SIRT1 in mammals, are NAD^+-dependent dacetylases. They influence transcription levels of numerous genes through the deacetylase activity, resulting in ribosomal DNA (rDNA) recombination and in extension of lifespan in response to calorie reaction (Imai, Johnson et al. 2000; Lin, Defossez et al. 2000). It has been reported that the NAD^+: NADH ratio is the critical factor in controlling in cellular activities rather than NAD^+ itself, and this ratio seems to depend on changes in NADH levels (Lin, Ford et al. 2004). PARP transfers ADP-ribose to target proteins such as histone, transcriptional factors and DNA repair proteins. PARP play important roles in DNA repair and genomic stability (de Murcia, Niedergang et al. 1997; Wang, Stingl et al. 1997). ART functions in the immune response and G protein-coupled signaling (Corda and Di Girolamo 2002).

B. 3. NAD^+ biosynthesis in *Saccharomyces cerevisiae*

In yeast, NAD^+ can be synthesized via the *de novo* pathway from tryptophan under aerobic conditions or aspartic acid and glutamic acid under anaerobic conditions (Panozzo, Nawara et al. 2002). Alternatively, NAD^+ is generated by recycling nicotinamide (NAM) via the salvage pathway (Lin and Guarente 2003). In the *de novo* pathway, tryptophan is converted to quinolinic acid, through several steps in which the products of the *BNA* genes are involved (Figure 2.A). Quinolinic acid enters into the

salvage pathway after it is converted to nicotinic acid mononucleotide (NaMN) by quinolinate phosphoribosyltransferase encoded by the *QPT1* gene.

In the salvage pathway, NaMN is adenylated to form nicotinic acid deamido-NAD (NaAD) by nicotinate mononucleotide adenyltransferase, encoded by the *NMA1* and *NMA2* genes. NaAD is converted to NAD^+ by NAD^+ synthase, encoded by the *QNS1* gene, an essential gene in yeast. NAD^+ is hydrolyzed by sirtuins, NAD^+-dependent protein deacetylases, such as Sir2p, Hst1p, Hst2p, Hst3p and Hst4p (Table 1). In the deacetylase reaction catalyzed by sirtuins, one molecule of NAD^+ is cleaved into ADP-ribose and NAM (Figure 2.A). The acetyl group derived from the acetylated amino-terminal tails of protein is transferred to ADP-ribose, forming 2'-O-acetyl-ADP ribose (Tanner, Landry et al. 2000; Tanny and Moazed 2001). NAM inhibits the activity of Sir2p, Hst1p and Hst2p, as a feedback mechanism (Landry, Slama et al. 2000; Bitterman, Anderson et al. 2002). NAM is deaminated into NA by nicotinamidase, encoded by the *PNC1* gene, which is homologous to the bacterial PncA gene (Ghislain, Talla et al. 2002). Alternatively, NA is imported from the growth medium by the high-affinity NA transporter, encoded by the *TNA1* gene. *TNA1* gene transcription is upregulated in response to absent or low NA concentration (Klebl, Zillig et al. 2000). NA is converted into NaMN by nicotinic acid phosphoribosyltransferase encoded by the *NPT1* gene. The formation of NaMN is the point of convergence of the *de novo* and salvage pathway for NAD^+ synthesis.

Recently, it was reported that, independently of NA and NAM, nicotinamide ribose (NR), originally derived from milk, can serve as a precursor of NAD^+ in yeast via the salvage pathway (Bieganowski and Brenner 2004)(Figure 2. A). NR is first converted into NMN (nicotinamide mononucleotide) by nicotinamide ribose kinase, encoded by the *NRK1* gene, and then to NAD^+ via a reaction catalyzed by the products of the *NMA1* and *NMA2* genes (Bieganowski and Brenner 2004; Belenky, Racette et al.

2007; Tempel, Rabeh et al. 2007) . Alternatively, NR is split into ribose and NAM by the hydrolase and phosphorylase encoded by the *URH1* and *PNP1* genes, respectively (Belenky, Racette et al. 2007). NR is transported from the medium by the NR transporter, encoded by *NRT1* (Belenky, Moga et al. 2008). The growth deficiency of *qns1*Δ mutant can be overcome by growing cells in medium containing NR (Bieganowski and Brenner 2004).

B. 4. NAD^+ biosynthesis in mammals

Mammalian NAD^+ synthesis is accomplished either through the *de novo* pathway from tryptophan or through the salvage pathway from exogenous NAM and NA (Figure 2.B). As in yeast, *de novo* synthesis of NAD^+ occurs by conversion of tryptophan into quinolinic acid through several steps. However, in mammals, in contrast to yeast, in the salvage pathway, NAM is recycled for NAD^+ synthesis, independent of NA. Another precursor, NR, described in the previous section, is involved in the NAM recycling pathway.

NAM is converted into nicotinamide mononucleotide (NMN) by NAM phosphoribosyltransferase (also known as: Nampt/PBEF/visfatin), which is the enzyme catalyzing the rate limiting step in NAD^+ metabolism in mammals (Samal, Sun et al. 1994; Fukuhara, Matsuda et al. 2005; Revollo, Grimm et al. 2007). Its structural gene, NAMPT, responds to stress and nutrients. When Nampt is overexpressed, cells show an increase in mitochondrial NAD^+ levels even during exposure to genotoxic agents (Yang, Yang et al. 2007). NMN is converted into NAD^+ by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (Nmnat), which is encoded by the NMNAT1, 2 and 3 genes.

Unlike yeast, mammals do not convert NAM directly into NA. Therefore, NA

enters mammalian NAD^+ metabolism only from dietary sources. NA is converted into NaMN by NA phosphoribosyltransferase (Naprt). NaMN is converted into NaAD by Nmnat and lastly into NAD^+ by NAD^+ synthase. Intracellular NAD^+ levels in human liver cells (HepG2 cells) are increased by additional dietary NA through Naprt (Hara, Yamada et al. 2007).

NAD^+ is utilized by Sirtuins, SIRT1 through 7. SIRT1 is a homolog of yeast Sir2p. SIRT1 participates in various cellular activities. For example, SIRT1 deacetylates PGC1α, a transcription coactivator, resulting in its activation. Deacetylated PGC1α induces upregulation of genes related to mitochondrial function and fatty acid utilization (Rodgers, Lerin et al. 2005). SIRT1 is also involved in repression of the activity of the tumor suppressor, p53 (Vaziri, Dessain et al. 2001), and regulation of transcription of stress-resistance genes by FOXO proteins (Brunet, Sweeney et al. 2004) (Luo, Nikolaev et al. 2001). SIRT1, SIRT3 and SIRT4 are related to fatty acid synthesis in mitochondria through activation of acetyl CoA synthetase (AceCS1 and 2) via the deacetylase activity of these sirtuins (Schwer, North et al. 2002; Haigis, Mostoslavsky et al. 2006; Hallows, Lee et al. 2006). SIRT3 and SIRT4 are homologous with CobB in *Salmonella* (Starai, Celic et al. 2002).

B. 5. Changes in NAD^+ metabolism affect the activity of sirtuins

NAD^+ dependent protein deacetylases, sirtuins, are involved in NAD^+ metabolism. In *Saccharomyces cerevisiae*, there are five Sir2 homologs, namely Sir2p and Hst1p, Hst2p, Hst3p and Hst4p (Table 1). In contrast, *Schizosaccharomyces pombe*, fission yeast, contains only three sirtuin homologs, Sir2p, Hst2p and Hst4p (Durand-Dubief, Sinha et al. 2007). Mammals have seven sirtuins, SIRT1 through SIRT7. Sir2p, which is the *S. cerevisiae* homolog of human SIRT1, plays a role in gene silencing at specific chromosomal sites, including telomeres, silent mating type

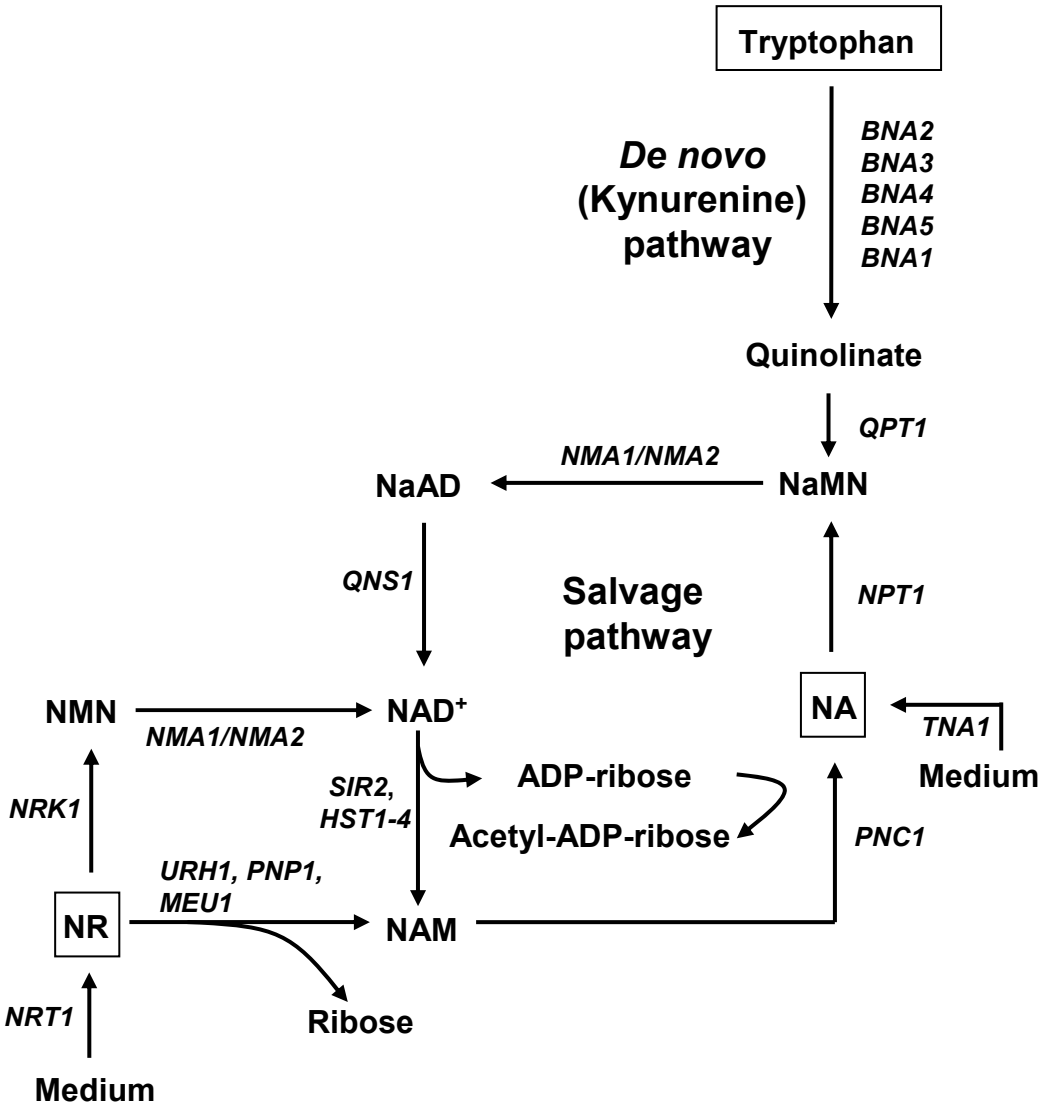


Figure 2.A. Pathway of NAD^+ metabolism in *Saccharomyces cerevisiae*. Tryptophan, NA and NR in boxes are precursors of NAD^+ synthesis in *Saccharomyces cerevisiae*. (Belenky, Racette et al. 2007). One molecule of NAD^+ is cleaved into ADP-ribose and NAM by sirtuins. The acetyl group derived from acetylated protein is transferred to ADP-ribose, forming 2'-O-acetyl-ADP-ribose. NAM inhibits sirtutins. Designations in italics refer to structural genes in NAD^+ metabolism. NaMN, nicotinic acid mononucleotide; NaAD, deamido-NAD; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NA, nicotinic acid; NR, nicotinamide ribose; NMN, nicotinamide mononucleotide.

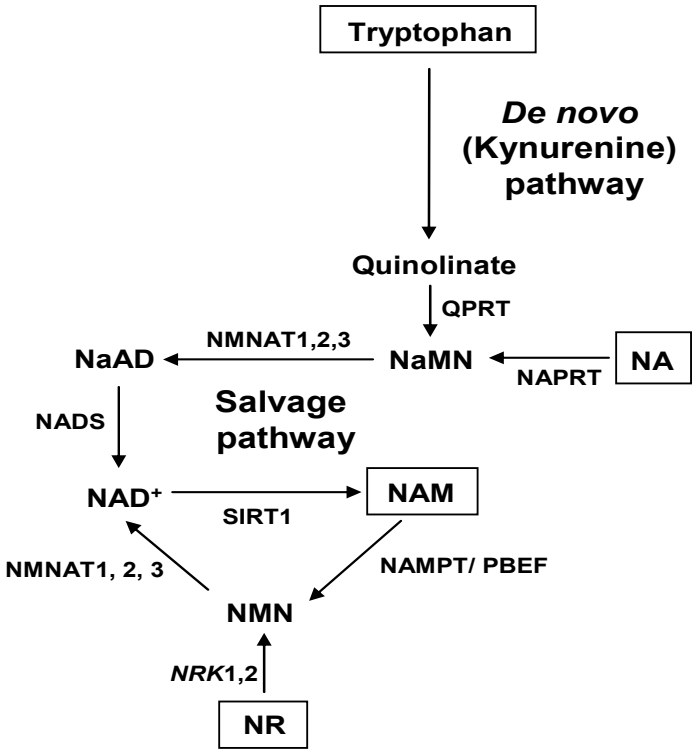


Figure 2.B. Pathway of NAD^+ metabolism in mammals. Tryptophan, NAM, NA and NR are precursors of NAD^+ synthesis. In *de novo* synthesis from tryptophan, several enzymatic steps are involved; tryptophan oxygenase, kynurenine formamidase, kynurenine-3 monooxygenase, kynureninase, 3-hydroxy anthranilate 3,4-dioxygenase and one non-enzymatic step. The salvage pathway includes those enzymes encoded by genes indicated above. Bold characters indicate metabolites in NAD^+ metabolism; NaMN, nicotinic acid mononucleotide; NaAD, deamido-NAD; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NA, nicotinic acid; NR, nicotinamide ribose; NMN, nicotinamide mononucleotide.

loci and rDNA (Rine and Herskowitz 1987; Gottschling 2000; Smith, Avalos et al. 2002). Sir2p activity is related to longevity under calorie restriction conditions (Lin, Defossez et al. 2000).

Hst1p is known as a sensor and regulator of cellular NAD^+ levels via the repression of *TNA1* and the *BNA* genes (except *BNA3*) (Bedalov, Hirao et al. 2003). Hst2p, in which is the *S. cerevisiae* homolog of human SIRT2, is a cytoplasmic protein and modulates nucleolar and telomere silencing (Perrod, Cockell et al. 2001). Hst3p and Hst4p in *S. cerevisiae* are involved in the cellular process such as DNA damage repair for maintenance chromosome stability and DNA replication (Celic, Masumoto et al. 2006; Pan, Ye et al. 2006).

The salvage pathway for NAD^+ synthesis seems primarily to exist in the nucleus, since Npt1p and Nam2p involved in the pathway are concentrated in the nucleus in yeast (Anderson, Bitterman et al. 2002).

Deletion of *NPT1* results in a decrease in Sir2p activity selectively at telomeres and in rDNA (Smith, Brachmann et al. 2000). The reason for decreased Sir2p activity may be related to changes in NAD^+ levels since the *npt1*Δ mutant exhibits lower NAD^+ levels than the wild type strain (Sandmeier, Celic et al. 2002; Smith, Avalos et al. 2002; Bedalov, Hirao et al. 2003). Furthermore, cells transformed with *NPT1, PNC1, NMA1* or *NMA2* on high copy plasmids show increased Sir2p-dependent silencing, but do have altered NAD^+ levels (Anderson, Bitterman et al. 2002). High copy expression of these genes seems to increase flux through the salvage pathway, resulting in enhanced Sir2p activity.

The *pnc1*Δ mutant, which exhibits normal NAD^+ levels, also shows silencing defects at the telomere and rDNA, presumably the result of accumulation of NAM, an inhibitor of sirtuins (Sandmeier, Celic et al. 2002; Gallo, Smith et al. 2004). Pnc1p levels measured by western blot are elevated in response to heat stress, hyperosmotic

shock, H_2O_2 exposure and DNA damage (Ghislain, Talla et al. 2002; Anderson, Bitterman et al. 2003; Gallo, Smith et al. 2004). Pnc1p is localized not only in the nucleus but also in the cytoplasm. Pnc1p localization is regulated by the external environment; Pnc1p is localized in the cytoplasm under the salt stress and amino acid restriction (Anderson, Bitterman et al. 2003).

The deletion of *BNA2,* involved in the *de novo* pathway, results in no change in Sir2p activity, although it results in decreased NAD^+ levels in cells grown in synthetic complete (SC) medium lacking tryptophan (Anderson, Bitterman et al. 2002; Bedalov, Hirao et al. 2003). However, it is important to point out that standard SC media contains 3.25 μM of nicotinic acid (NA) and 10 μM of inositol (Sherman 1991).

NAD^+ from the salvage pathway appears to have a more critical effect on the activity of sirtuins than NAD^+ derived from *de novo* synthesis, perhaps because the proteins of salvage pathway are primarily concentrated in the nucleus, whereas proteins involved in *de novo* synthesis are evenly distributed throughout the nucleus and cytoplasm (Anderson, Bitterman et al. 2002; Sandmeier, Celic et al. 2002).

Mutations in NAD^+ metabolism affect sirtuins activity by influencing NAD^+ levels or the rate of NAD^+ recycling as described above. How do mutations in sirtuins themselves affect NAD^+ levels? Unexpectedly, no changes in NAD^+ level were observed in several sirtuin mutants, as compared to wild type, implying that sirtuins are not major consumers of NAD^+ (Smith, Brachmann et al. 2000; Anderson, Bitterman et al. 2002).

As stated above, wild type strains exhibit reduced NAD^+ levels when grown in SC medium lacking tryptophan compared to cells grown in SC medium containing tryptophan. The sirtuin mutants, *sir2*Δ*, hst2*Δ*, hst3*Δ and *hst4*Δ, have wild type NAD^+ levels in SC medium lacking tryptophan (Bedalov, Hirao et al. 2003). In contrast to wild type, NAD^+ levels in the *hst1*Δ mutant was not reduced when cells were grown in

SC medium lacking tryptophan as compared with the levels observed in SC medium containing tryptophan because Hst1p is a regulator of the cellular NAD^+ levels. Moreover, Hst1p has a low affinity for NAD^+, consistent with its role as a sensor of NAD^+ levels (Bedalov, Hirao et al. 2003). Deletion of *HST2* results in very low NAD^+-dependent histone deacetylase activity in whole-cell extracts, whereas other sirtuin mutants have normal deacetylase activity (Smith, Brachmann et al. 2000).

Table 1. Functions and locations of each sirtuin in *Saccharomyces cerevisiae*

Siruins	Location	Functions	References
Sir2p	Nucleus	Silencing at telomeric, rDNA and mating loci, longevity, genomic stability	(Lin, Defossez et al. 2000)
Hst1p	Nucleus	Sensor and regulator of NAD^+ levels, repression of middle sporulation genes in vegetative growth	(Bedalov, Hirao et al. 2003)
Hst2p	Cytoplasm, depending on the cell cycle	Affects life span independent of Sir2p, major deacetylase activity among sirtuins in vitro, nucleolar and telomeric silencing	(Perrod, Cockell et al. 2001)
Hst3p	Nucleus	Chromosome stability by binding the origin of replication of 2μm plasmid, mitotic recombination	(Grunweller and Ehrenhofer-Murray 2002)
Hst4p	Nucleus	Chromosome stability, mitotic recombination	(Pan, Ye et al. 2006)

NAD^+ levels are also dependent on growth conditions (Sandmeier, Celic et al. 2002). In the wild type strain grown in YPD medium, NAD^+ levels are higher than in

cells grown in SC medium. The *bna1*Δ mutant grown in YPD medium has NAD^+ levels similar to the wild type NAD^+ levels, but in SC medium, the mutant exhibits decreased NAD^+ levels compared with wild type NAD^+ levels. Supplementation with additional NA in SC media to a level of 10 μM NA results in recovery of NAD^+ levels in the *bna1*Δ strain to levels comparable to wild type cells grown in YPD. However, the *npt1*Δ mutant exhibits low NAD^+ levels in all growth conditions (i.e., in both YPD and SC media). These data imply that the increase in NAD^+ levels occurring in response to exogenous NA are due to NAD^+ synthesis via the salvage pathway. It is clear that mutations in both the *de novo* and the salvage pathways affect NAD^+ levels and NAD^+ levels are also influenced by growth conditions, including supplementation with NA and tryptophan. However, the relationship of cellular NAD^+ levels to various sirtuin activities is clearly complex.

C. Concluding remarks

In this study, I examined the interaction of inositol biosynthesis and NAD^+ metabolism. I found that the presence of phospholipid precursor inositol in the growth medium alters NAD^+ levels as well as expression levels of genes involved in NAD^+ metabolism. *BNA2* expression levels in wild type cells were increased, especially in the absence both of inositol and NA, a growth condition resulting low NAD^+ levels. I found that the *npt1*Δ mutant, defective in NAD^+ metabolism via the salvage pathway, exhibits inositol auxotrophy (Ino^- phenotype) at 37°C. The Ino^- phenotype is generally associated with lower levels of *INO1* expression and/or alterations in phospholipid metabolism (Henry and Patton-Vogt 1998). The *npt1*Δ mutant also exhibited choline sensitivity in the absence of inositol, consistent with observations of other categories of mutants with Ino^- phenotypes. NA starvation restored *INO1* expression levels in *npt1*Δ cells to wild type levels, but the Ino^- phenotype of *npt1*Δ was only partially

rescued under these conditions. The *npt1*Δ mutant exhibited consistently low NAD^+ levels under all conditions tested. However, *BNA2* expression levels in *npt1*Δ cells responded to both inositol and NA. *BNA2* expression reached its highest level with 3 hrs of transfer of cells to I-NA+ medium. Interestingly, the deletion of *HST1* or the addition of NAM to the medium enhanced the Ino^- phenotype of *npt1*Δ, suggesting that the sirtuin, Hst1p, might play a role in cell viability in the absence of inositol and at higher growth temperatures. My data indicate that increased NAD^+ levels and sirtuin activity are necessary for lipid homeostasis and cell viability under conditions of stress created by high temperature and the absence of inositol.

CHAPTER TWO
MATERIALS and METHODS

A. Strains and growth conditions

A. 1. Strains : All strains used this study were derived from the S288C purchased from Research Genetics (Invitrogen). The strains are listed in Table 2. Strains were maintained on YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose).

A. 2. Construction of *opi1*Δ*npt1*Δ, *pnc1*Δ*npt1*Δ, *sir2*Δ*npt1*Δ, *hst1*Δ*npt1*Δ and *tna1*Δ*npt1*Δ mutants : Complete disruption of the *NPT1* gene was performed by PCR-mediated gene replacement as in described by Longtine, Mckenzie *et al*.(1998) in *opi1*Δ, *pnc1*Δ, *sir2*Δ, *hst1*Δ and *tna1*Δ strains. pRS313 containing *HIS3* was used for as a PCR template. The PCR fragment was constructed using a NPT1-F1 primer (5' ATA TTT TAG AAG AAA GGT GGA TTT AGC CTT TTG TAC CTA TTC GCG CGT TTC GGT GAT GAC GGT G 3') and NPT1-R1 primer (5' ATC GCA GAG ATG TCA GTA CCC ATA ACT GAA TAA TTA GGT CGA TTT TGC CGA TTT CGG CCT ATT GG 3'). The PCR product includes 40 nucleotides of homology to the target sequence. The entire *NPT1* open reading frame in each strain was replaced with the *HIS3* gene. Mutants were selected on His- dropout media.

A. 3. Growth conditions : Standard synthetic complete (SC) medium contains 10 μM inositol and 3.25 μM nicotinic acid (Sherman 1991). However, all experiments in this study were carried out in synthetic complete growth media (SC) containing 75 μM inositol (I+) or lacking inositol (I-) in the presence or absence of 3.25 μM

nicotinic acid (NA+ or NA-). Cells were continuously grown in four different growth media, i.e., medium containing inositol and NA (I+ NA+), lacking inositol and containing NA (I- NA+), containing inositol and lacking NA (I+ NA-) and lacking inositol and NA (I- NA-) at 30^{o}C.

Table 2. List of strains used in this study

Strain	Genotype	Source
BY 4742	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Research Genetics
SLY30	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *sir2*::KanMX	Research Genetics
LSY40	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *bna1*::KanMX/ MATa , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *bna1*::KanmMX	Research Genetics
SLY41	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *hst1*::KanMX	Research Genetics
SLY42	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *pnc1*::KanMX	Research Genetics
SLY43	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *npt1*::KanMX	Research Genetics
SLY44	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *bna2*::KanMX	Research Genetics
SLY50	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *hst1*::KanMX n*pt1*::HIS3	Research Genetics
SLY56	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *tna1*::KanMX	Research Genetics
SLY57	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *nma1*::KanMX	Research Genetics
SLY58	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *nma2*::KanMX	Research Genetics
SLY59	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *opi1*::KanMX	Research Genetics
SLY60	MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 *opi1*::KanMX n*pt1*::*HIS3*	This study
SLY61	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *sir2*::KanMX *npt1*::*HIS3*	This study
SLY62	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *pnc1*::KanMX *npt1*::*HIS3*	This study
SLY63	MATα *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *tna1*::KanMX n*pt1*::*HIS3*	This study
SH114	MAT*a ade1 ino1-13*/ MATα *ade1 ino1-13*	Henry Lab
SJY425	MATα , *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *ino1::HIS3*	Jesch. S.A.

B. Phenotypic assay

B. 1. Analysis of inositol auxotrophy (Ino$^-$ phenotype) : Strains were grown overnight in medium containing 75 μM inositol and 3.25 μM NA (I+ NA+) or containing inositol without NA (I+ NA-), washed and resuspended at A_{600}=1.0/mL and used to conduct 4 sequential 1:10 fold serial dilutions in sterile distilled water. The concentration of NA was kept consistent during growing in pre-culture and on plates. Each dilution was spotted on SC medium containing 75 μM inositol or lacking inositol with or without 3.25 μM of NA (i.e., I+ NA+, I- NA+, I+ NA-, I- NA-). To test other compounds potentially influencing the Ino$^-$ phenotype of mutants, 1 mM choline (C), 5 mM nicotinamide (NAM), or isonicotinic acid (INA) were added to SC medium described above (i.e., I+ NA+ C+ or I- NA+ C+, or I+ NA+ NAM+ or I- NA+ NAM+, or I+ NA+ INA+ or I- NA+ INA+). In other cases a specific amino acid, such as tryptophan or threonine, was eliminated from SC medium (i.e., I+ NA+ Trp- or I- NA+ Trp-, or I+ NA+ Thr- or I- NA- Thr-). The plates were incubated at 30 and 37ºC for 5 days.

B. 2. Construction of a high copy plasmid carrying the *HST1*: In this study, the *HST1* high copy plasmid was constructed using a 2,311 bp genomic fragment, containing the *HST1* open reading frame plus ~500 bp upstream of the start codon and ~300 bp downstream of the stop codon. The fragment was amplified by PCR using the following primers; HST1-F (5' CG GGATCC GGT AGC AAT GAC ACG TTA AG 3') and HST1-R (5'GC GGATCC CAA GAG GAA CTG ATC AGT GG 3') using *Pfx* proofreading DNA polymerase (Invitrogen). The PCR fragment was digested using *Bam*HI and inserted into the *Bam*HI site of pRS426 (*URA3*, 2μ). The plasmid was transformed into DH5α *E.coli* cells using chemically competent cells (Seidman, Struhl et al. 2001) and purified using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmid

DNA was introduced into yeast cells using the High Efficiency Transformation Method as described by Gietz and Woods (2002).

B. 3. Suppressor analysis of the Ino$^-$ phenotype of the *npt1*Δ mutant : All plasmids used for suppressor analysis are described in Table 3. Other highcopy plasmids carrying genes, except *HST1* and *SIR2*, previously known to suppress Ino$^-$ phenotype of specific mutants listed in Table 3 were available in the laboratory.

Table 3. List of plasmids used for suppressor analysis of Ino$^-$ phenotype

Plasmids	Gene	Replicon	Marker	reference
pPS65	*ACC1-794*	*CEN*	*URA3*	(Shirra, Patton-Vogt et al. 2001)
pPS47	*SNF4-204*	*CEN*	*URA3*	(Shirra and Arndt 1999)
YEp351-*SIR2*	*SIR2*	2μ	*LEU2*	Obtained from L.Pillus
pRS426-*HST1*	*HST1*	2μ	*URA3*	This study
pRS426-*NTE1*	*NTE1*	2μ	*URA3*	(Murray and McMaster 2005)

C. Measurement of relative transcript abundance

C. 1. RNA isolation : Cells were grown to mid-logarithmic phase (A_{600}=0.5~0.6) in I+ medium with or without NA (I+ NA+ or I- NA+) and then shifted to I- medium with or without NA (I- NA+ or I- NA-) at 30 ºC or 37^{o}C. Cells were harvested by filtration immediately following the media shift (0 hr) and were subsequently harvested at various intervals as specified in individual experiments out to 9 hrs. Alternatively, under continuous growth conditions, cells were allowed to reach mid-logarithmic phase (A_{600}=0.5~0.6) and then were harvested at several intervals from 0 hr to 9 hrs as observed in specific experiments. In such experiments,

the NA concentration was kept constant as cells were shifted from I+ to I- medium (from I+ NA+ to I- NA+ or from I+ NA- to I- NA-). Harvested cells were flash frozen and stored at -80 ºC.

Total RNA was obtained from harvested cells by the hot phenol acid method (Kohrer and Domdey 1991). 1 µg of total RNA was fractionated on 1.1% glyoxal agarose gels and transferred to Nytran SuPercharge nylon membrane (Whatman, Stanford, ME) in 20X SSC (Sodium chloride Sodium Citrate). Strand-specific ^{32}P-labeled riboprobes were synthesized from the linerized template probe plasmid by *in vitro* transcription according to the manufacturer's instructions (Promega) using SP6 RNA polymerase (Invitrogen) or T7 RNA polymerase (Fermentas). The template for each gene probe was constructed from a fragment containing part of the ORF of each gene amplified by PCR, which was inserted into the pGEM1 plasmid. Membranes were hybridized with *INO1*, *BNA2,* or *TNA1* and *ACT1* probes in formamide hybridization buffer and washed at a final stringency of 0.2X SSC with 0.1% SDS (Sodium Dodecyl Sulfate) at 60ºC. Gene expression levels (mRNA levels) were normalized to *ACT1* mRNA levels. Quantification was performed by analysis on a Storm 860 PhosphorImager and analyzed with ImageQuant software (GE Healthcare, Piscataway, NJ).

C. 2. Construction of probes : Probes used in this study are listed in Table 4. A fragment (759 bp) of the *BNA2* gene was constructed using PCR with the following primers: BNA2-F (5'CG AAGCTT ACC ACT ACC AGT GCT AGA AA 3'), which contains *Hind*III site, and BNA2-R (5' GC CCTGCA CCT AAC ACC CTG TGG TAA CC3'), which contains *Pst*I site. After digestion with *Hind*III and *Pst*I restriction enzymes, the fragment was inserted into the pGEM1 (2865 bp) plasmid. A fragment (700 bp) of the *TNA1* gene was constructed by PCR using the following primers;

TNA1-F (5' CG AAGCTT GTC ACG TTC CAG GAA GAT G 3'), which contains *Hind*III site, and TNA1-R (5' GA CCTGCA GCA ACC CGA AGG CAT AGA ATG 3'), which contains *Pst*I site. After digestion with *Hind*III and *Pst*I, the fragment was inserted into the pGEM1 (2865 bp) plasmid. Other probes as listed in Table 4 were available in the laboratory from reviews and studies.

Table 4. List of probes used in this study

Plasmids	Gene/Restriction site/Promoter	Reference
pSPACT	*ACT1*/ *Sma*I/ SP6	(Chang, Jesch et al. 2004)
pJH310-*INO1*	*INO1*/ *Hind*III/ T7	(Hirsch and Henry 1986)
pBNA2	*BNA2*/ *Hind*III/ T7	This study
pTNA1	*TNA1*/ *Hind*III/ T7	This study

D. Intracellular metabolite analysis

D. 1. Analysis of intracellular NAD^+ level

D. 1. 1. Growth conditions : Cells were continuously grown in four different growth media (I+ NA+, I- NA+, I+ NA- and I- NA-) until A_{600}=1.0 in 500 ml cultures at 30 or 37°C. Cells were harvested by filtration and stored at -80 ºC.

D. 1. 2. Extraction of intracellular NAD^+ : Harvested cells were extracted with 250 µl of ice-cold 1M formic acid saturated with butanol for 30 min. 62.5 µl of 100% trichloroacetic acid (TCA) was added and samples were precipitated on ice for 15 min. Samples were centrifuged for 5 min and the acid soluble supernatants were collected. After the pellets were washed with 125 µl of 20% TCA and centrifuged, supernatants were added to the supernatants obtained from the 100% TCA extraction (Cornell and Veech 1983; Smith, Avalos et al. 2002).

D. 1. 3. Analysis of intracellular NAD^+ levels : 10 µl of 5 mg/ml alcohol dehydrogenase, 840 µl of Tris-lysine Buffer (pH 9.7) and 150 µl of extract were combined and incubated for 5 min at room temperature to allow NAD^+ to be converted into NADH. Reacted samples were detected at absorbance 340 nm (Smith, Avalos et al. 2002; Belenky, Racette et al. 2007). Moles of NAD^+ in the extract were calculated from NAD^+ standard curves. The standard curve was generated using commercial NAD^+ from Fluka followed by the reaction method described above. The concentration range of NAD^+ was between 0.25 to 1 mM. The absorbance of each experimental sample was used to calculate NAD^+ concentration using the standard curve.

D. 2. Analysis of intracellular nicotinic acid (NA) level

D. 2. 1. Growth conditions : For these experiments, pre-cultures (50 ml) were prepared overnight and shifted to new fresh 1L medium adjusted to A_{600}=0.2. When cultures reached A_{600}=1.0, cells were harvested by filtration. A final O.D. of A_{600}=1.0 was used, as in the samples collected for NAD^+ measurement. Harvested cells were washed with sterilized water and then stored at -80 ^oC.

D. 2. 2. Extraction of NA from cells : NA was extracted from each sample by the acid extraction method of Abramov *et al*. (Abramov Sh, Kotenko et al. 2003). 10% perchloric acid was added to the washed harvested cells, which were then placed on ice for 30 min. After centrifuging for an additional 5 min, supernatants were collected and titrated with saturated potassium chloride (KCl). After centrifuging for 15 min, supernatants were collected and applied to a C_{18} column (Supelco Co. Ltd., LC-18 SPE tubes). Prior to use, this column was activated with 1 ml of methanol and 1 ml of water. 1 ml of the extract was loaded on the column and eluted using 0.5 ml of 85% methanol applied for 4 times. However, only the second elution was collected since

NA was contained only in that elution fraction. This sample was purified by a nylon syringe filter (13 mm, 0.2 μm, Alltech Co. Ltd. Catalog NO. 2166) for preparing HPLC samples.

D. 2. 3. Analysis of intracellular NA by HPLC : A reverse phase column, C_{18} (Dionex Co. Ltd) was used and HPLC (Waters Co. Ltd) was run using a binary solvent system (Solvent A and B). Solvent A was mixture of 0.05 M ammonium acetate and methanol (99:1). Solvent B was 50% methanol. Before using the column, it was washed with a solvent mixture consisting of dichrolomethane and acetone (2:1). The flow rate of the HPLC solvent was 0.7 ml/min. The ratio of solvents A and B was changed over time as follows; A:B= 95:5 for 0-5 min, A: B=0:100 for 5-18 min, A: B= 0:100 for 18-20 min and the A:B= 95:5 for 20-30 min. The amount of nicotinic acid was calculated from the area of the peak and compared with the standard curve. The standard curve was constructed using the commercial NA (Sigma Co. Ltd). Total intracellular NA levels were normalized to the total cellular mass (μg of NA /mg of dried cells) per 1 L culture.

D. 3. Lipid analysis

D. 3. 1. Growth conditions : To analyze phospholipids and neutral lipids, cells were grown two different ways. Cells were grown continuously in a specific medium or cells were shifted from the presence of inositol to the absence of inositol keeping NA concentration constant. Cells were grown continuously in the presence or absence of 75 μM inositol with or without 3.25 μM NA (i.e., I+ NA+, I- NA+, I+ NA- and I- NA-) with 1μCi/mL of [1-^{14}C] acetate overnight. The cultures were then diluted to A_{600}=0.1 in the same media containing 1 μCi/mL of [1-^{14}C] acetate. When they reached at A_{600}=1, cells were harvested by filtration. Alternatively, cells were grown in the presence of inositol with or without NA (i.e., I+ NA+ or I+ NA-) with 1 μCi/mL of

[1-^{14}C] acetate for overnight. The cultures were transferred to fresh media maintaining the NA and inositol concentrations, diluted to A_{600}=0.1 containing 1 μCi/mL of [1-^{14}C] acetate. At mid-logarithmic phase (A_{600}=0.5~0.6), half of each culture was switched to medium lacking inositol keeping NA concentration and [1-^{14}C] acetate constant. The other half of each culture was allowed to continue growing in the original medium (i.e., I+ NA+ or I- NA+) with [1-^{14}C] acetate. When each culture reached A_{600}=1, it was harvested for lipid extraction.

D. 3. 2. Extraction of lipid from cells : 5 ml of each culture was mixed with 50% TCA (tricarboxylic acid). After 30 min incubation on ice, cells were pelleted and washed with distilled water. The pellet was extracted with 5 ml of chloroform: methanol (2:1 v/v) at 60 ^{o}C for 1hr. And then, 0.5 ml of 0.6% NaCl was added and mixed totally. The organic phase of the extraction was transferred to a new vial and evaporated under N_2 gas flow. The samples were resuspend with 0.5 ml of chloroform: methanol (2:1 v/v), and a fraction of this was used to quantify counts per minute (CPM) using a scintillation counter.

D. 3. 3. Separation of phospholipids and neutral lipids : Polar lipids (i.e., phospholipids) were separated by high-performance thin layer chromatography (HPTLC) on Whatman Silica Gel 60A plates using chloroform/ethyl acetate/acetone/isopropanol/ethanol/methanol/water/acetic acid (30:6:6:6:16:28:6:2, v/v/v/v/v/v/v/v) (Weerheim, Kolb et al. 2002). Hexane /diethylether/ formic acid (80:20:2 v/v/v) was used to analyze neutral lipids (Christie 2003). Quantitation was performed by analysis on a Storm 860 PhosphorImager (Amersham Biosciences) and analyzed with ImageQuant software. Metabolites were identified by comparison to known standards.

CHAPTER THREE
RESULTS

A. Interaction of inositol and NAD^+ metabolism

A. 1. Inositol auxotrophy (Ino^- phenotype) of mutants involved in NAD^+ metabolism

Inositol auxotrophy of mutants with defects in NAD^+ metabolism, *pnc1*Δ, *npt1*Δ, *nma1*Δ, *nma2*Δ, *sir2*Δ, *hst1*Δ, *bna2*Δ, *bna1*Δ, *tna1*Δ, as well as some double mutant strains, *pnc1*Δ*npt1*Δ, and *hst1*Δ*npt1*Δ was assessed. Growth in the presence or absence of 75 μM inositol (I+ or I-) was tested on plates in the presence or absence of 3.25 μM nicotinic acid (NA), (medium designation: I+ NA+ or I- NA+), at 30 and 37°C (Figure 3). All single and double mutants investigated were able to grow at 30°C whether inositol was present or not (Figure 3). However, at 37°C, the growth of several strains was impaired, especially in the absence of inositol. Among the single mutants, the *npt1*Δ strain exhibited an Ino^- phenotype at 37 °C (Figure 3.A). The *tna1*Δ mutant grew poorly at 37°C, even in medium containing inositol. The *tna1*Δ*npt1*Δ strain did not grow at all at 37°C, even in medium containing inositol (Figure 3.B).

The *pnc1*Δ and *sir2*Δ mutations had little any effect the Ino^- phenotype of the *npt1*Δ mutant (Compare growth of the *pnc1*Δ*npt1*Δ and *sir2*Δ*npt1*Δ strains in the absence of inositol at 37°C to *npt1*Δ in Figure 3.B). In contrast, the *hst1*Δ*npt1*Δ strain had a stronger Ino^- phenotype than the *npt1*Δ strain (Figure 3.B). The *opi1*Δ mutation completely suppressed the Ino^- phenotype of the *npt1*Δ mutant (i.e., the *opi1*Δ*npt1*Δ mutant grew well in I- NA+ medium at 37°C) (Figure 3.B).

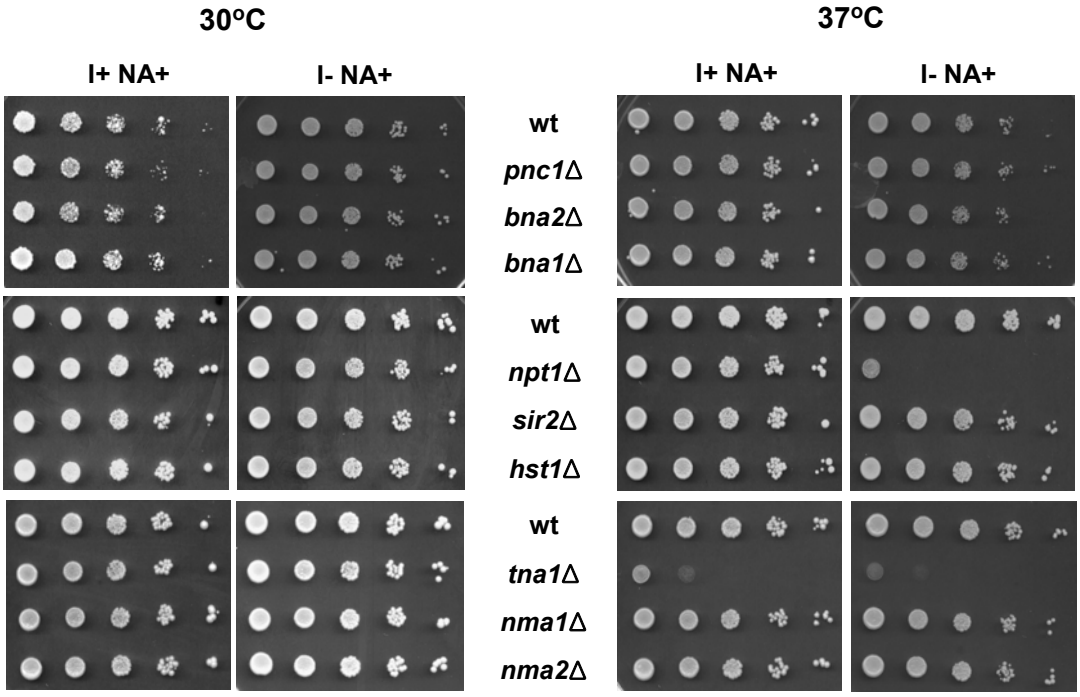


Figure 3.A. Ino$^-$phenotype of mutants involved in NAD$^+$ metabolism. Cells takenfrom cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions as described in the Materials and Methods and were spotted on plates containing or lacking 75 μM inositol in the presence of 3.25 μM NA (I+ NA+ and I- NA+ media, respectively). Plates were incubated for 5 days at 30 and 37°C.

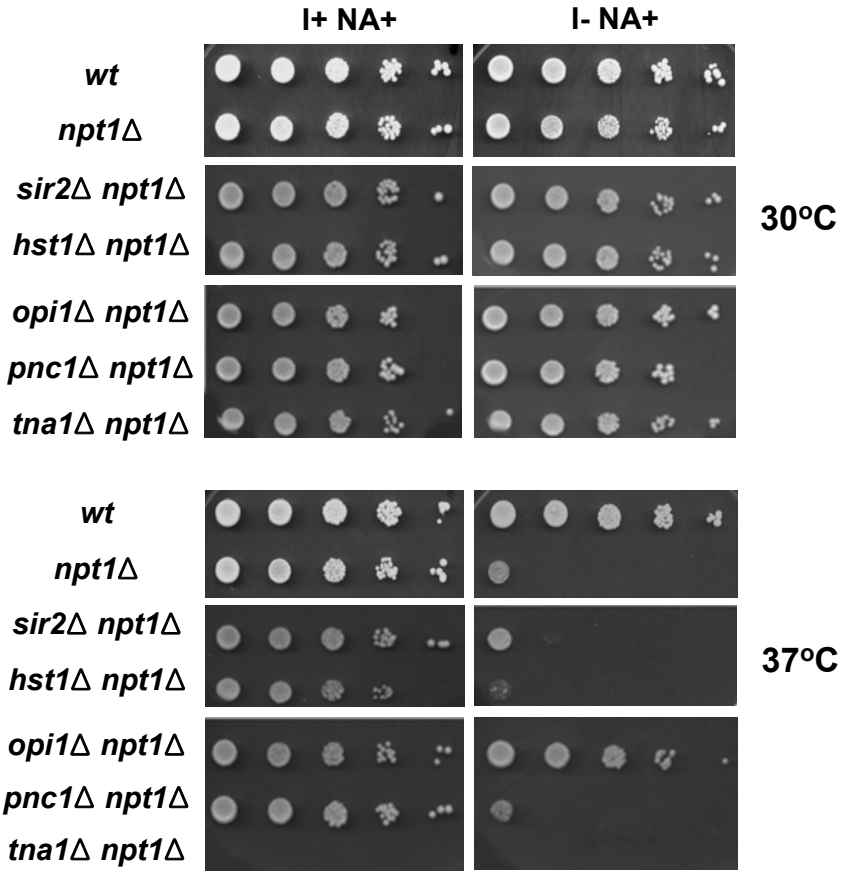


Figure 3.B. Ino$^-$ phenotype of double mutants involved in NAD$^+$ metabolism. Cells taken from cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions as described in the Materials and Methods and were spotted on plates containing or lacking 75 μM inositol in the presence of 3.25 μM NA (I+ NA+ and I- NA+ media). Plates were incubated for 5 days at 30 and 37^{o}C.

Elimination of NA from the medium partially rescued the Ino$^-$ phenotype of the *npt1*Δ mutant as well as that of double mutants, *pnc1*Δ*npt1*Δ and *hst1*Δ*npt1*Δ. However, the absence of NA resulted in diminished growth of *pnc1*Δ cells in the absence of inositol at 37^{o}C (Figure 4).

Nicotinamide (NAM), a metabolite in the NAD$^+$ pathway (Figure 2.A), has been shown to be an inhibitor of the activity of sirtuins such as Sir2p, Hst1p and Hst2p (Landry, Slama et al. 2000; Gallo, Smith et al. 2004; Sauve, Moir et al. 2005). Addition of 5 mM NAM to the medium caused diminished growth of the *npt1*Δ, *pnc1*Δ*npt1*Δ and *hst1*Δ*npt1*Δ strains, even in the presence of inositol at 37^{o}C (Figure 5). The *npt1*Δ strain, especially, did not grow well if NAM was present, even at 30^{o}C in medium containing inositol (Figure 5).

Choline is a major precursor of phospholipid metabolism (Figure 1.C). Addition of choline to growth medium containing inositol causes a further reduction in *INO1* expression levels and its presence in medium lacking inositol has been shown to enhance the Ino$^-$ phenotype of certain mutants (Nunez, Jesch et al. 2008). Addition of 1 mM choline to I- medium resulted in strengthening of the Ino$^-$ phenotype of the *npt1*Δ mutant compared to cells grown in I- medium lacking choline at 37^{o}C (Figure 5). Also, the Ino$^-$ phenotype of the *pnc1*Δ*npt1*Δ and *hst1*Δ*npt1*Δ strains was exacerbated when choline was present in the growth medium at 37^{o}C. However, choline did not affect the phenotype of these strains tested at 30^{o}C (Figure 6).

Tryptophan is a precursor to NAD$^+$ synthesis in *de novo* pathway (Figure 2.A). Therefore, the phenotype of mutants involved in NAD$^+$ metabolism might be influenced by tryptophan in the medium, although all of these strains are able to synthesize tryptophan *de novo*. The absence of tryptophan partially suppressed on the Ino$^-$ phenotype of the *pnc1*Δ*npt1*Δ and *hst1*Δ*npt1*Δ strains at 37^{o}C (Figure 6).

The effect of threonine on diploid homozygous mutants having an Ino$^-$

phenotype has been studied in our laboratory (Mannuel Villa., unpublished data, 2008). In these experiments, removal of threonine from the medium resulted in a weak Ino$^-$ phenotype at 30^{o}C in strains containing the *npt1*Δ mutation, including *npt1*Δ, *pnc1*Δ*npt1*Δ and *hst1*Δ*npt1*Δ, and resulted in a stronger Ino$^-$ phenotype at 37^{o}C. However, the *pnc1*Δ and *hst1*Δ mutants did not have an Ino$^-$ phenotype at 30 or 37^{o}C whether threnoine was present in medium or not (Figure 8).

Summary : The *npt1*Δ mutant exhibits an Ino- phenotype at 37^{o}C which is strengthened in the presence of choline, threonine and NAM and partially suppressed by removal of NA from the medium. This phenotype is strengthened when the *hst1*Δ mutation is introduced into the *npt1*Δ strain.

Table 5. Effect of NA and NAM on Ino$^-$ phenotype at 37^{o}C

	Control		NA effect		NAM effect	
	I+ NA+	I- NA+	I+ NA-	I- NA-	I+ NA+ NAM+	I- NA+ NMA+
wt	+++	+++	+++	+++	+++	+++
***npt1*Δ**	+++	**+**	+++	**++**	**+**	**–**
***pnc1*Δ**	+++	+++	+++	++	+++	+++
***Hst1*Δ**	+++	+++	+++	+++	+++	+++
***pnc1*Δ*npt1*Δ**	+++	+	+++	++	+	–
***hst1*Δ*npt1*Δ**	+++	**–**	+++	+	**–**	**–**

Table 6. Effect of choline on Ino$^-$ phenotype at 37^{o}C

	Control		Choline effect	
	I+ NA+	I- NA+	I+ NA+ C+	I- NA+ C+
Wt	+++	+++	+++	+++
***npt1*Δ**	+++	**+**	+++	**–**
***pnc1*Δ**	+++	+++	+++	+++
***hst1*Δ**	+++	+++	+++	+++
***pnc1*Δ*npt1*Δ**	+++	+	+++	**–**
***hst1*Δ*npt1*Δ**	+++	**–**	+++	**–**

Scale: +++, grew well (dilutions 4-5); ++, grew (dilutions 2-3); +, grew (dilution 1); -,no growth.

Figure 4. Effect of NA on the Ino$^-$ phenotype of several mutants at 30 and 37^{o}C. Cells taken from cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions as described in the Materials and Methods and were spotted on plates containing or lacking 75 μM inositol in the absence of NA (I+ NA- and I- NA-) compared with the medium containing or lacking inositol in the presence of 3.25 μM NA (I+ NA+ and I- NA+). Plates were incubated for 5 days at 30 and 37^{o}C.

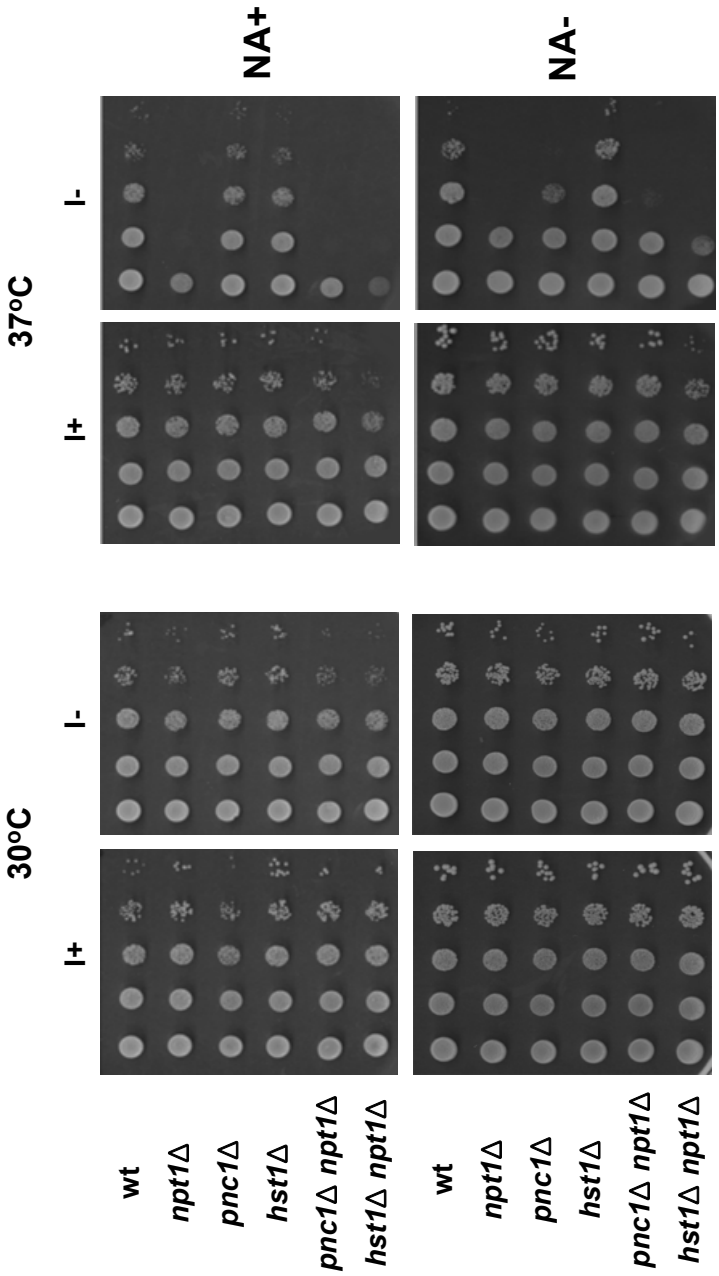

NA+
NA-
37°C
30°C
I+
I-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ

Figure 5. Affect of NAM on the Ino$^-$ phenotype of mutants at 30 and 37^{o}C. Cells taken from cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions and were spotted on plates containing or lacking 75 μM inositol with 3.25 μM NA (I+ NA+ NAM-and I- NA+ NAM-) or addition of 5 mM nicotinamide (NAM) (I+ NA+ NAM+ and I- NA+ NAM+) and in the absence of NA with 5mM NAM (I+ NA- NAM+ and I- NA- NAM+). Plates were incubated for 5 days at 30 and 37^{o}C.

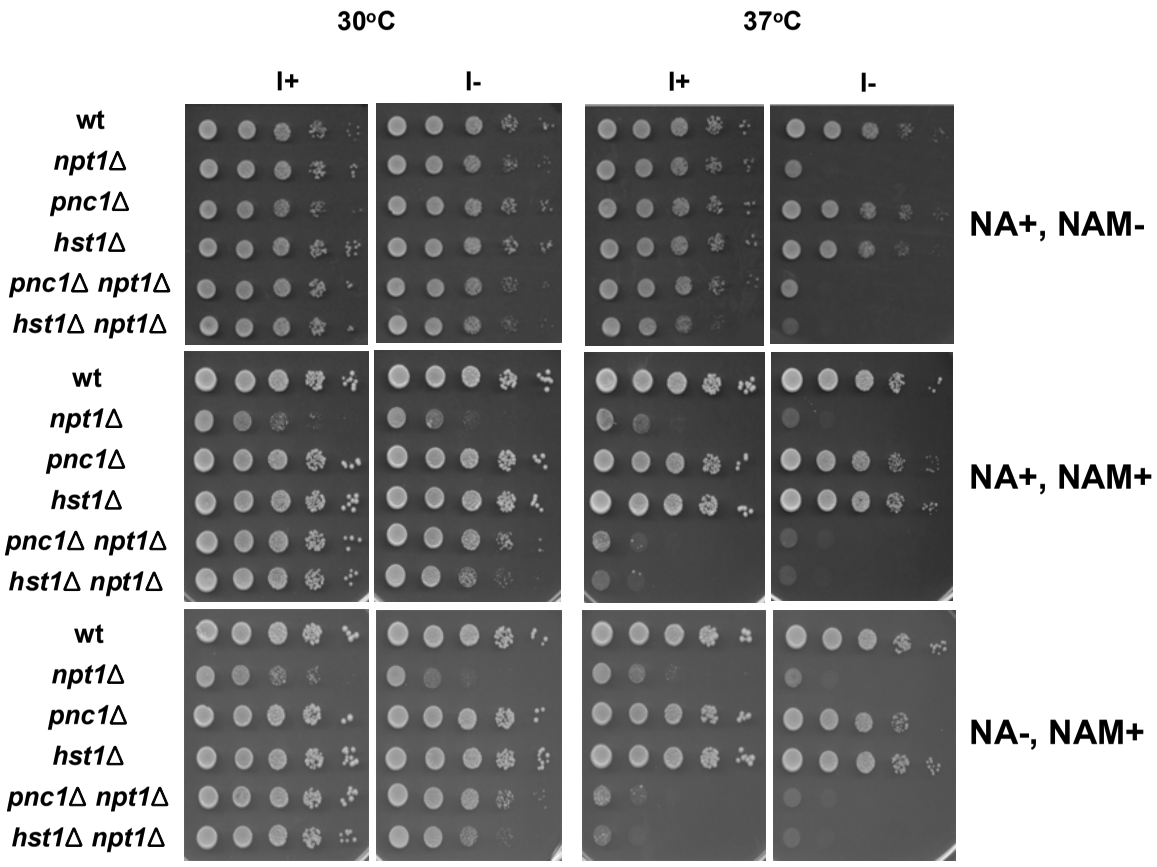

30°C
37°C
I+
I-
I+
I-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, NAM-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, NAM+
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA-, NAM+

Figure 6. Effect of choline on the Ino$^-$ phenotype of mutants at 30 and 37^{o}C. Cells taken from cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions and were spotted on plates containing or lacking 75 μM inositol with 3.25 μM NA (I+ NA+ Cho- and I- NA+ Cho-) or addition of 1 mM choline (I+ NA+ Cho+ and I- NA+ Cho-). Plates were incubated for 5 days at 30 and 37^{o}C.

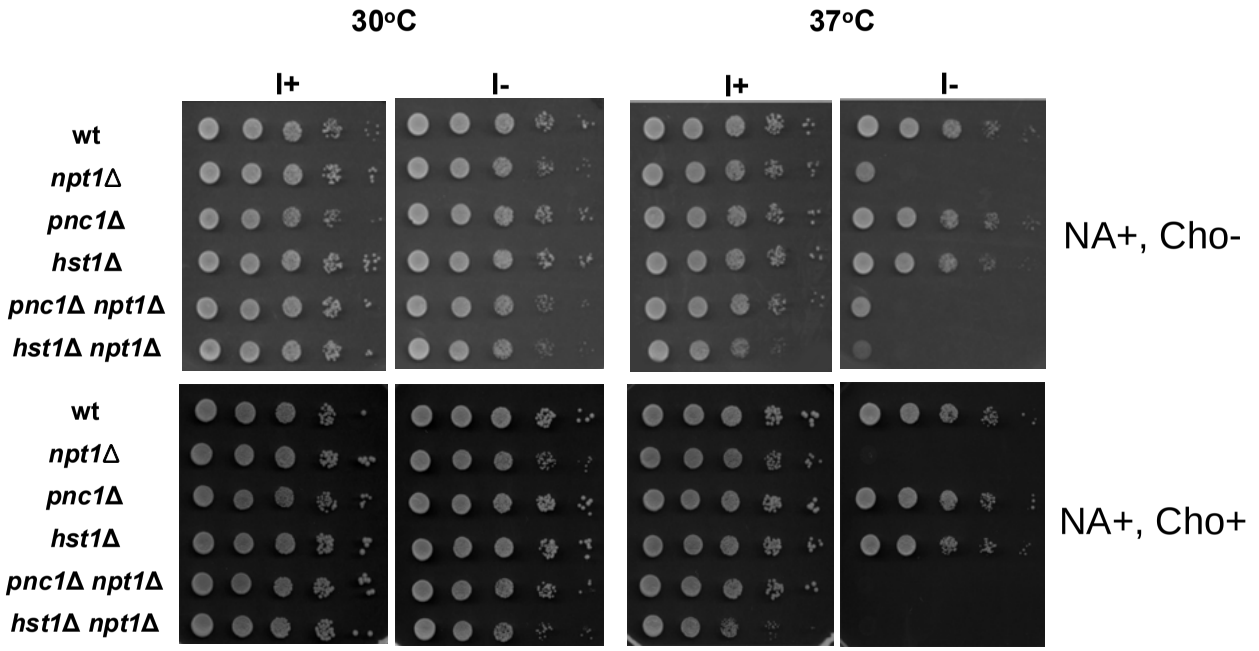

30°C
37°C
I+
I-
I+
I-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, Cho-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, Cho+

Figure 7. Effect of tryptophan on the Ino$^-$ phenotype of several mutants at 37^{o}C. Cells taken from cultures at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions as described in the Materials and Method and were spotted on plates containing or lacking 75 μM inositol with 3.25 μM NA and tryptophan (I+ NA+ Trp+ and I- NA+ Trp+) or without tryptophan (I+ NA+ Trp- and I- NA+ Trp-). Plates were incubated for 5 days at 30 and 37^{o}C.

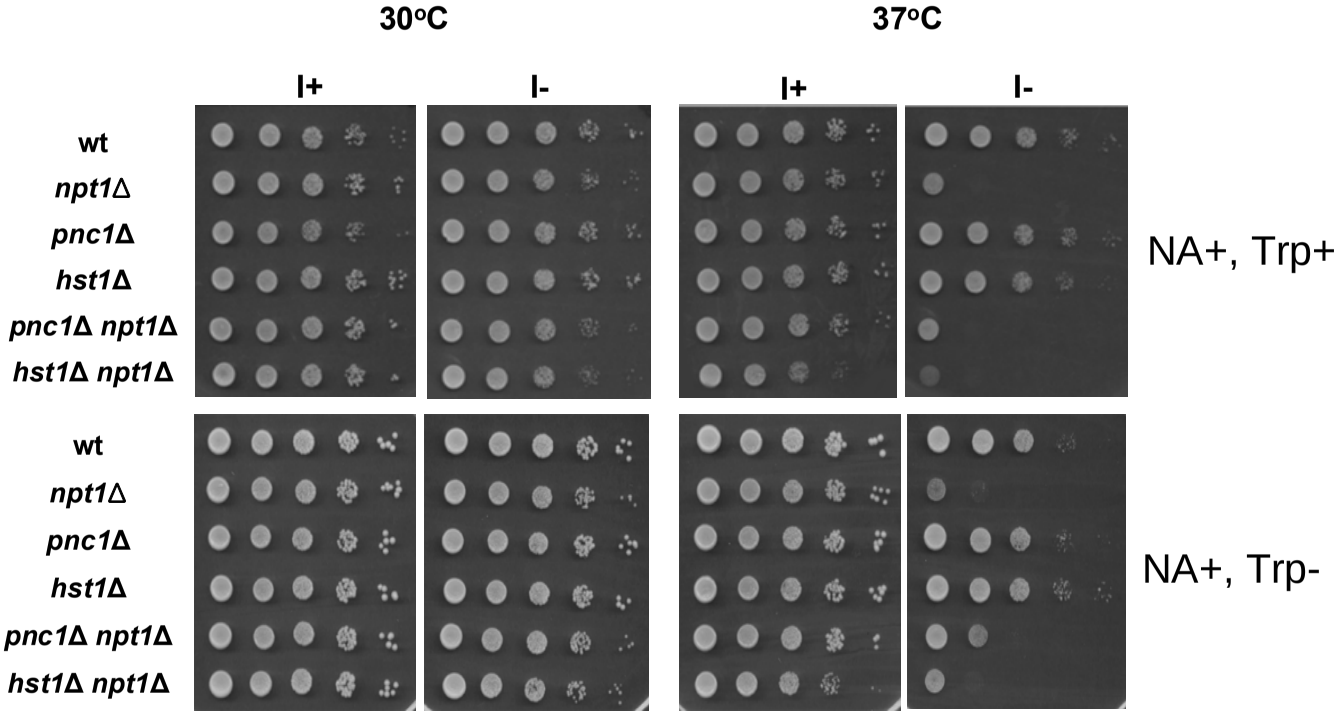

30°C
37°C
I+
I-
I+
I-
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, Trp+
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ
NA+, Trp-

Figure 8. Effect of threonine on Ino$^-$ phenotype of several mutants at 37^{o}C. Cells taken from culture at 1.0 A_{600}/ml were subjected to 4 sequential 1:10 serial dilutions as described in the Materials and Methods and were spotted on plates containing or lacking 75 μM inositol with 3.25 μM NA and threonine (I+ NA+ Thr+ and I- NA+ Thr+) or without threonine (I+ NA+ Thr- and I- NA+ Thr-). Plates were incubated for 5 days at 30 and 37^{o}C.

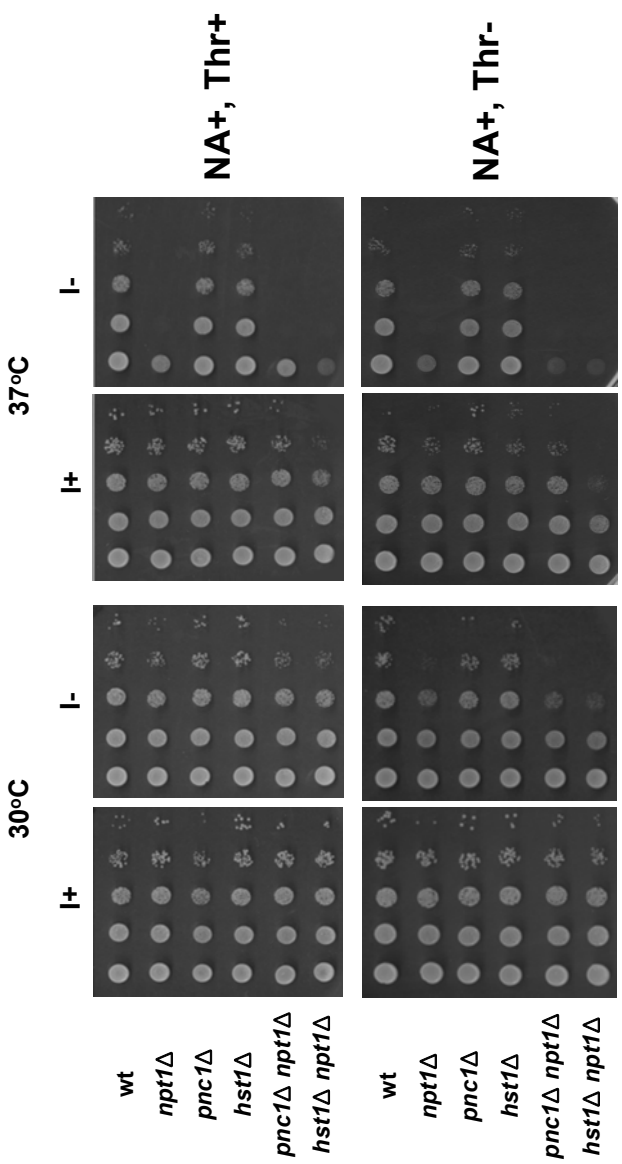

NA+, Thr+
NA+, Thr-
37°C
30°C
I-
I+
wt
npt1Δ
pnc1Δ
hst1Δ
pnc1Δ npt1Δ
hst1Δ npt1Δ

Table 7. Effect of tryptophan and threonine on Ino$^-$ phenotype at 37^{o}C

	Control		Tryptophan effect		Threonine effect	
	I+ NA+	I- NA+	I+ NA+ Trp-	I- NA+ Trp-	I+ NA+ Thr-	I- NA+ Thr-
wt	+++	+++	+++	+++	+++	+++
***npt1*Δ**	+++	+	+++	+	+++	+
***pnc1*Δ**	+++	+++	+++	+++	+++	+++
***hst1*Δ**	+++	+++	+++	+++	+++	+++
***pnc1*Δ*npt1*Δ**	+++	+	+++	++	+++	−
***hst1*Δ*npt1*Δ**	+++	−	+++	+	++	−

Scale: +++, grew well (dilutions 4-5); ++, grew (dilutions 2-3); +, grew (dilution 1); -,no growth.

A. 2. Suppression analysis of the Ino$^-$ phenotype of the *npt1*Δ mutant using high copy plasmids containing the *HST1* and *SIR2* genes involved in NAD$^+$ metabolism

The *SIR2* and *HST1* genes encode sirtuins, which are involved in the NAD$^+$ metabolism, and play important roles in utilizing or regulating NAD$^+$ for transcriptional regulation (Bedalov, Gatbonton et al. 2001; Bedalov, Hirao et al. 2003). The introduction of the *hst1*Δ mutation into an *npt1*Δ strain resulted in a stronger Ino$^-$ phenotype in the double mutant in comparison to the *npt1*Δ mutant alone (Figure 3.B). The effect high copy expression of *SIR2* and *HST1* on the Ino$^-$ phenotype of the *npt1*Δ mutant was also tested. High copy plasmids carrying either *SIR2* or *HST1* did not appear to influence the Ino$^-$ phenotype of the *npt1*Δ strain. However, unexpectedly, when the empty high copy plasmids, pRS425 (*LEU2*, 2μ) and pRS426 (*URA3*, 2μ), were introduced into *npt1*Δ cells as a control, the strain exhibited a growth defect in medium lacking inositol at 30^{o}C (Figure 9.A and B), while the untransformed *npt1*Δ strain had no Ino$^-$ phenotype at 30^{o}C and only exhibited inositol auxotrophy at 37^{o}C (Figure 3.A). Thus, it appeared that carrying a high copy plasmid with no insert reduced the growth of *npt1*Δ cells on I- medium at 30^{o}C. This effect was not seen in wild type cells transformed with the plasmid. A potential explanation for this effect

may be that Hst3p, one of sirtuins involved in NAD^+ salvage pathway, binds to the origin of DNA replication of high copy plasmids (Grunweller and Ehrenhofer-Murray 2002). Therefore, the consistently low NAD^+ levels in *npt1*Δ cells (Figure 17) might decrease the activity of Hst3p, resulting in loss of stability of DNA replication of high copy 2μ plasmid used in these experiment.

Summary : The presence of *HST1* or *SIR2* on the high copy plasmids at 30°C in I- medium dose not influence the Ino^- phenotype resulting from the presence of plasmid. While this result is difficult to interpret, but it is clear that the Ino^- phenotype is not influenced by expression of the *SIR2* or *HST1* genes on the plasmid.

A. 3. Plasmids containing genes involved in lipid metabolism, *CEN-ACC1-794, CEN- SNF4-204* and 2μ-*NTE1* plasmids.

Known suppressors of the Ino^- phenotypes of other well studied mutants were tested. One such suppressor is a dominant mutation in the *ACC1* gene (Shirra, Patton-Vogt et al. 2001). The *ACC1* gene encodes acetyl coenzyme A carboxylase (Acc1p), which catalyzes the rate-limiting step in fatty acid synthesis (Woods, Munday et al. 1994). *ACC1-794* is a dominant partial loss of function mutation which results in reduced Acc1p activity both *in vivo* and *in vitro* (Shirra, Patton-Vogt et al. 2001). *ACC1-794* was isolated as a suppressor of the Ino^- phenotype of the *snf1*Δ mutant. The *SNF1* gene encodes a protein kinase required for glucose repression. Snf1p is a homologue of the mammalian AMP dependent kinase (Carling, Aguan et al. 1994). *ACC1-794* is a dominant suppressor presumably because Acc1p exists in cells as a multimer. The presence of one *ACC1-794* subunit in the Acc1p complex presumably impairs the activity of the overall enzyme (Shirra, Patton-Vogt et al. 2001).

Acc1p is phosphorylated and negatively regulated by Snf1p kinase. The *INO1* gene cannot be derepressed when Acc1p activity levels are high. The Ino^- phenotype

of *snf1*Δ cells is thought to be the result of very high Acc1p activity levels. Thus, *ACC1-794* suppresses the Ino$^-$ phenotype of the *snf1*Δ mutant by partially inhibiting Acc1p activity. Inhibition of Acc1p activity leads to *INO1* expression in the absence of functional Snf1p kinase activity (Shirra, Patton-Vogt et al. 2001). *SNF4-204* is a dominant suppressor of a number of mutants having Ino$^-$ phenotypes, such as the *spt15-328* and *spt15-341* mutants, which are partially defective in the TATA binding protein (Shirra and Arndt 1999). The *SNF4* gene encodes the regulatory subunit of the Snf1p kinase. Snf1p is activated by binding Snf4p. The *snf4*Δ mutation has an Ino$^-$ phenotype similar to *snf1*Δ, due to failure to phosphorylate Acc1p. The *SNF4-204* mutation enhances the physical interaction between Snf1p and Snf4p leading to higher Snf1p activity and thus lowers Acc1p activity which allows *INO1* to be expressed (Shirra and Arndt 1999). The ability of both of these dominant mutations (i.e., *ACC1-794* and *SNF4-204*) on *CEN* plasmids (i.e., low copy plasmids) to suppress Ino$^-$ phenotypes of various mutants is therefore likely to be related to the reduction of Acc1p activity. Transformation with low copy *CEN*-plasmids containing *ACC1-794* or *SNF4-204* did not influence Ino$^-$ phenotype of the *npt1*Δ mutant (Figure 10.A). In this test, unlike empty high copy plasmids pRS425 (*LEU2*, 2μ) and pRS426 (*URA3*, 2μ), the low copy *CEN*-empty vector, pRS316 (*URA3*) did not affect the growth of *npt1*Δ cells grown in medium lacking inositol at 30^{o}C.

The *NTE1* gene has also been identified as a high copy suppressor of the Ino$^-$ phenotype of certain mutants, such as the *mpk1*Δ mutant, defective in the Cell Wall Integrity Protein Kinase C (CWI-PKC) stress response pathway (Nunez, Jesch et al. 2008). Nte1p was identified as an ER associated phospholipase B responsible for the turnover of phosphatidylcholine (PC) in both yeast and mammalian cells. This enzyme deacylates PC, generating intracellular soluble glycerophosphocholine (GroPC) and free fatty acids (FFA) (Zaccheo, Dinsdale et al. 2004) (Figure 1.C). Nte1p mediated

turnover of PC is stimulated in the presence of choline or at higher growth temperatures such as 37°C (Dowd, Bier et al. 2001; Zaccheo, Dinsdale et al. 2004; Gaspar, Aregullin et al. 2006). In this experiment, at 30°C, transformation with empty high copy plasmids resulted in an Ino⁻ phenotype in the *npt1*Δ strain similar to the observations shown in Figure 8.A and B. However, the presence of the *NTE1* gene on the plasmid appears to suppress the Ino⁻ phenotype caused by the empty high copy plasmid at 30°C as well as partially suppress the Ino⁻ phenotype associated with the *npt1*Δ mutation at 37°C (Figure 10.B).

Table 8. Summary of the effects of plasmids carrying potential suppressors on the Ino⁻ phenotype of the *npt1*Δ mutant at 30°C and 37°C

strains	Plasmids used	Phenotype at 30°C	Phenotype at 37°C
WT	**Empty-pRS426**	+++	+++
***npt1*Δ**	**Empty-pRS316**	+++	+
***npt1*Δ**	**Empty-pRS426**	-	-
***npt1*Δ**	**Empty-pRS425**	+	-
***npt1*Δ**	**pRS425-2μ-*SIR2***	+	-
***npt1*Δ**	**pRS426-2μ-*HST1***	+	-
***npt1*Δ**	***CEN-ACC1-794***	+++	-
***npt1*Δ**	***CEN-SNF4-204***	+++	-
***npt1*Δ**	**pRS426-2μ-*NTE1***	+++	++

Scale: +++, well grew (dilutions 4-5); ++, grew (dilutions 2-3); +, less grew (dilution 1); -,no growth.

Summary : High copy *NTE1* partially suppresses the Ino⁻ phenotype of the *npt1*Δ mutant at 37°C (Figure 10.B).

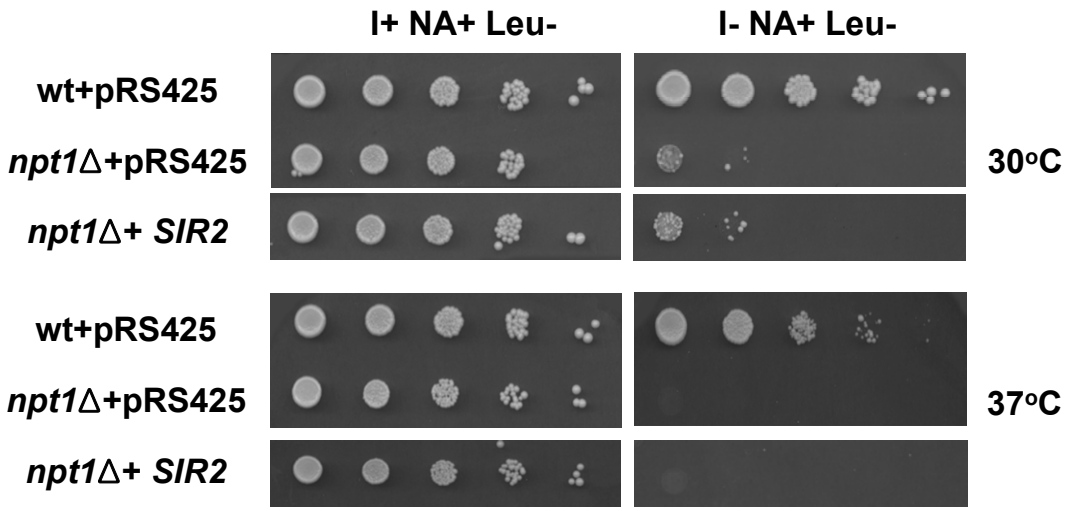


Figure 9.A. Analysis of Ino$^-$ phenotype of the *npt1*Δ mutant transformed with a high copy plasmid carrying the *SIR2* gene. Cells were grown in Leu- medium for overnight culture and then spotted on plates containing (I+) or lacking inositol (I-) with NA (NA+) in the absence of leucine (I+ NA+ Leu-, I- NA+ Leu-, I+ NA- Leu- and I- NA- Leu-). Plates were incubated for 5 days at 30 and 37^{o}C. As a control, wild type and *npt1*Δ strains were transformed with the empty vector, pRS425 (*LEU2*, 2μ) as a control. The high copy plasmid pRS425 carrying *SIR2* was introduced into the *npt1*Δ strain.

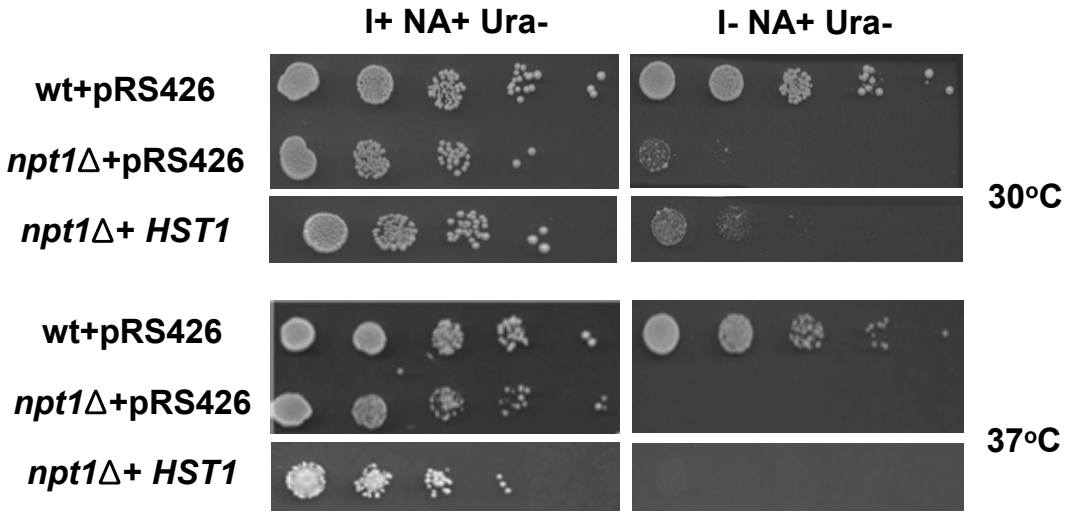


Figure 9.B. Analysis of Ino$^-$ phenotype of the *npt1*Δ mutant transformed with a high copy plasmid carrying the *HST1* gene. Cells were grown in Ura- medium for overnight culture and then spotted on plates containing (I+) or lacking inositol (I-) with NA (NA+) in the absence of uracil (I+ NA+ Ura-, I- NA+ Ura-, I+ NA- Ura- and I- NA- Ura-). Plates were incubated for 5 days at 30 and 37^{o}C. As a control, wild type and *npt1*Δ strains were transformed with the empty vector, pRS426 (*URA3*, 2μ) as a control. The high copy plasmid pRS426 carrying *HST1* was introduced into the *npt1*Δ strain.

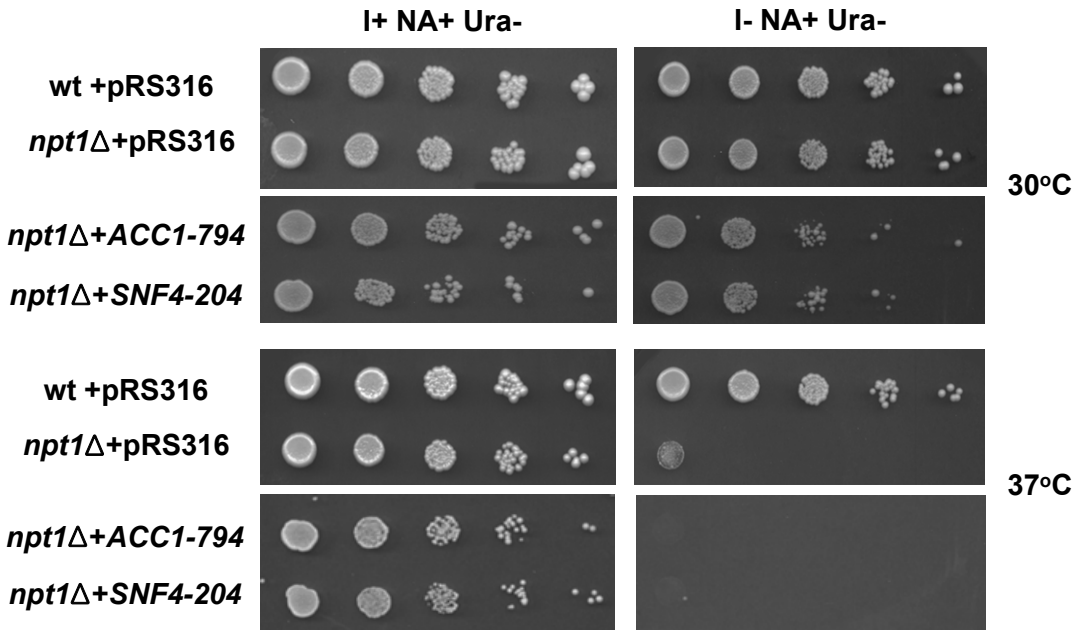


Figure 10.A. Analysis of the Ino$^-$ phenotype of the *npt1*Δ mutant transformed with the *CEN-ACC1-794*, *CEN-SNF4-204* plasmids. Cells were grown in Ura- medium for overnight culture and then spotted on the plates containing or lacking inositol and NA in the absence of uracil (I+ NA+ Ura-, I- NA+ Ura-, I+ NA- Ura- and I- NA- Ura-). Plates were incubated for 5 days at 30^{o}C. Wild type and *npt1*Δ strains were transformed with empty vector, pRS316 (*URA3*, *CEN*) as a control. *CEN-ACC1-279* and *SNF4-204* plasmids were introduced into the *npt1*Δ strain.

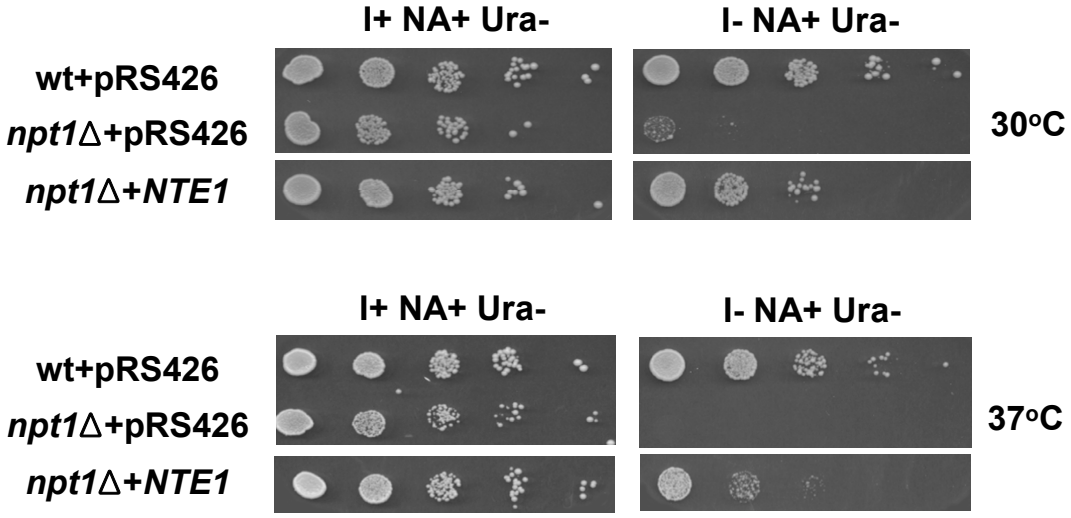


Figure 10.B. Analysis of Ino$^-$ phenotype of the *npt1*Δ mutant transformed with high copy *NTE1* plasmid. Cells were grown in Ura- medium for overnight culture and then spotted on the plates containing or lacking inositol and NA in the absence of uracil (I+ NA+ Ura-, I- NA+ Ura-, I+ NA- Ura- and I- NA- Ura-). Plates were incubated for 5 days at 30^{o}C. Wild type and *npt1*Δ strains were transformed with empty vector, pRS426 (*URA3*, 2μ) as a control. The *NTE1* high copy plasmid was introduced into the *npt1*Δ strain.

A. 4. Effect of exogenous inositol, NA and temperature on *INO1* expression levels in the wild type strain

Transcription of the *INO1* gene is regulated both by inositol availability and growth stage (Hirsch and Henry 1986; Carman and Henry 1989; Jiranek, Graves et al. 1998). *INO1* expression is repressed in the presence of inositol and derepressed in the absence of inositol. The presence of 10 μM inositol results in slightly reduced *INO1* expression in logarithmically growing cells and 75 μM inositol results in 10-fold or greater repression of *INO1* compared with cells grown under fully derepressed condition, i.e., in the absence of inositol, (Hirsch and Henry 1986). Growth stage has also been shown to affect *INO1* expression (Lamping, Luckl et al. 1994; Jiranek, Graves et al. 1998). Cells at the mid-logarithmic phase of growth in the absence of inositol exhibit the highest levels of *INO1* transcript but transcript abundance declines as cells enter late logarithmic and stationary phase.

To determine how *INO1* expression responds to NA availability and to explore the relationship between the regulation of inositol biosynthesis and NAD^+ metabolism, it was necessary to examine *INO1* transcript abundance in cells in various phases of growth. Initial experiments were aimed at determining whether *INO1* transcription was regulated in the wild type strain in response to NA. Cells were grown continuously at 30^{o}C in the presence of inositol with or without NA and were shifted at mid-logarithmic phase (A_{600}=0.5-0.6) to medium lacking inositol (i.e., from I+ NA+ to I- NA+ or from I+ NA- to I- NA-). As expected, immediately after the shift to new medium at 0 hr, *INO1* was repressed, reflecting the effect of prior growth in the presence of inositol (Figure 11). At subsequent time points after the shift to medium lacking inositol, *INO1* expression gradually increased, as seen by 1hr, and had peaked by 3 hrs. After 3 hrs, *INO1* expression levels gradually decreased as the culture progressed after 9 hrs, into late-logarithmic phase. *INO1* expression levels in wild type

cells grown in the absence of NA (shifted from I+ NA- to I- NA-) were similar to the *INO1* levels observed in the presence of NA (from I+ NA+ to I- NA+) (Figure 11).

Since the Ino$^-$ phenotype was only clearly seen in *npt1*Δ cells grown in I- NA+ medium at 37^{o}C, additional experiments were aimed at determining whether *INO1* transcriptional regulation in the wild type strain was responding to more than one factor, such as the combination of nutrients as well as temperature. Such an experiment is shown in Figure 11, experiment 4 in which *INO1* expression was followed after a shift from I+ NA+ at 30^{o}C to I- NA- at 37^{o}C (experiment 4 in Figure 12). Wild type cells were also grown in medium containing inositol and NA (I+ NA+) at 30^{o}C until mid-logarithmic phase and shifted to, I- NA+ medium at 30^{o}C (experiment 1 in Figure 12),or I- NA+ at 37^{o}C (experiment 2 in Figure 12), or I- NA- medium at 30^{o}C (experiment 3 in Figure 12). Alternatively, cells were shifted from I+ NA+ at 30^{o}C to I- NA- medium at 37^{o}C (experiment 4 in Figure 12); In summary, experiment 1 in Figure 11 tests the effect of inositol availability. Experiment 2 in Figure 12 tests the effect of inositol availability and temperature. Experiment 3 in Figure 12 tests the effect of inositol and NA availability. Experiment 4 in Figure 12 tests the effect of inositol, NA availability and temperature. *INO1* expression levels of experiment 1 in Figure 12 serve as a control for comparison to the levels in the other conditions, in which multiple variables are changed (experiments 2, 3 and 4 in Figure 11). The *INO1* expression levels were decreased significantly when cells were shifted to I- media at 37^{o}C compared to 30^{o}C (compare experiment 1 to experiment 2). Likewise, *INO1* expression levels were decreased if cells were shifted to I- NA- medium compared to I- NA+ medium (compare experiment 1 to experiment 3). When temperature and NA availability were both changed (i.e. cells were shifted to I- NA- medium at 37^{o}C, experiment 4), the effects appeared additive (compared experiment 4 to experiment 2 and 3)

Summary : The shift to media lacking inositol has the greatest overall effect on *INO1* expression. However, higher temperature and lack of NA independently result in the reduction of derepression of *INO1* in cells shifted to I- medium. The presence of NA and higher temperature both contributed to the decrease in *INO1* expression after derepression.

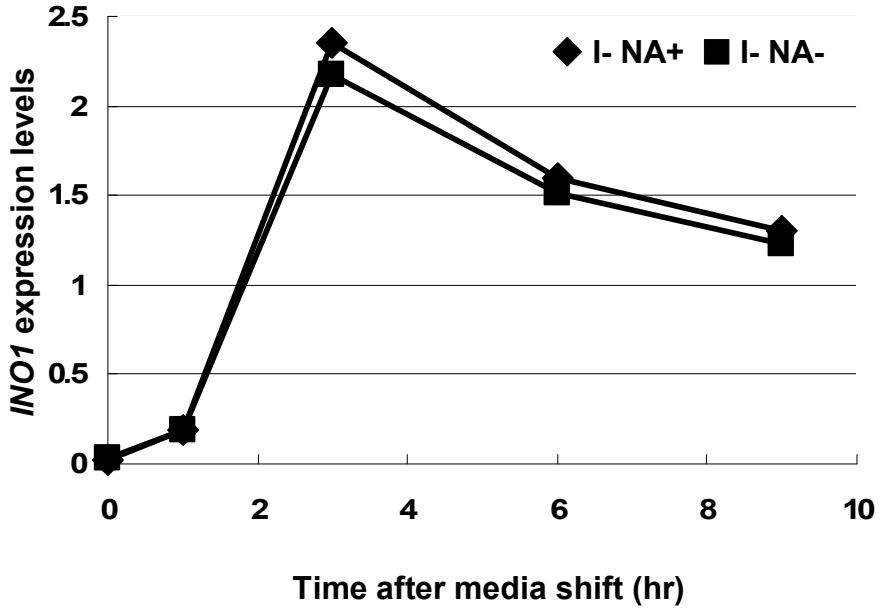


Figure 11. *INO1* expression patterns in the wild type strain following a shift to I- NA+ or I- NA- medium at 30°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA-) until mid-logarithmic phase at 30°C. Cells were then shifted to inositol free (I-) medium, while leaving NA concentration unchanged (i.e., I+ NA+ → I- NA+ or I+ NA- → I- NA-). Samples were harvested at 0, 1, 3, 6, and 9 hrs following the media shift. *INO1* mRNA levels were normalized to *ACT1* mRNA levels. One experiment representative of at least three repetitions is shown.

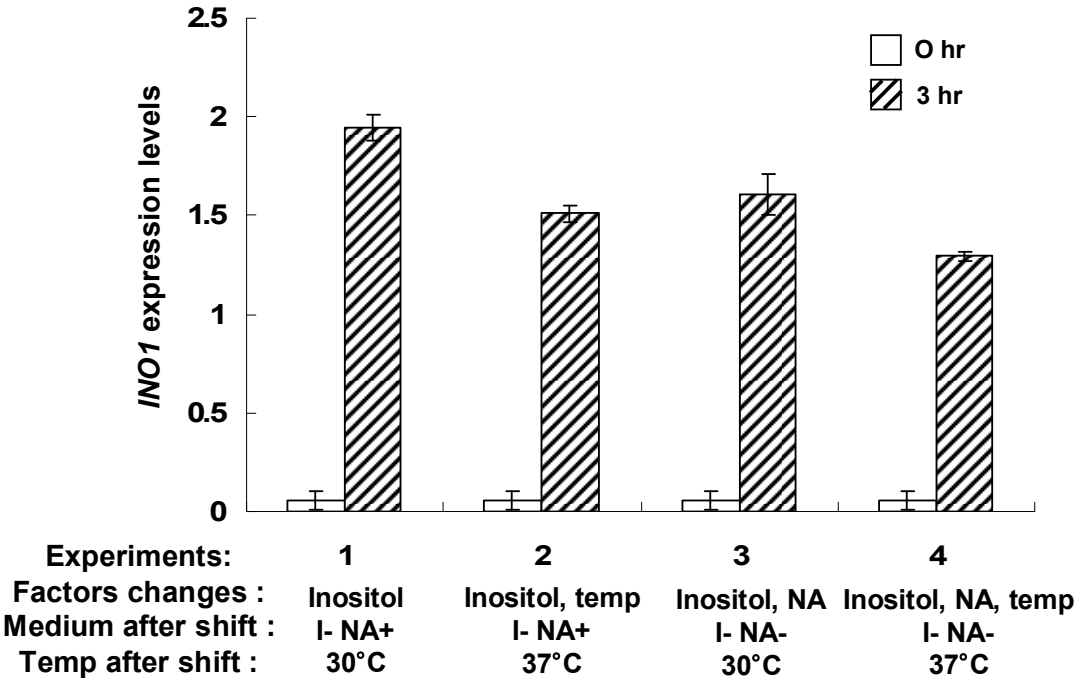


Figure 12. NA availability and temperature affect *INO1* expression levels in the wild type strain. Cells were grown in media containing inositol and NA (I+ NA+) until mid-logarithmic phase at 30^{o}C. Cells were then shifted to the growth conditions stated in the figure. *INO1* expression levels were measured at 0 and 3 hrs after the shift to a new as following growth condition, to I- NA+ at 30^{o}C (1), to I- NA+ at 37^{o}C (2), to I- NA- at 30^{o}C (3), to I- NA- at 37^{o}C (4). *INO1* mRNA levels were assessed by quantitative Northern blot analysis and normalized to *ACT1* mRNA levels as described in the Materials and Methods. Data are averages from three independent experiments, and the error bars represent the standard deviation (S.D), n=3.

A. 5. Effect of inositol and NA on *INO1* expression levels in the *npt1*Δ strain

As described previously and shown in Figure 1, the *npt1*Δ mutant has an Ino$^-$ phenotype at 37^{o}C and the phenotype was partially rescued by elimination of nicotinic acid (NA) from the growth medium (Figure 4). Ino$^-$ phenotypes are usually associated with insufficient *INO1* transcription, lowered Ino1p activity and/or other alterations in phospholipid metabolism (Henry and Patton-Vogt 1998). The design of the experiments involving *npt1*Δ was identical to that used for wild type cells. I measured *INO1* expression levels in the *npt1*Δ mutant following a shift from medium containing inositol to medium lacking inositol and with or without NA at both 30 and 37^{o}C to determine whether the Ino$^-$ phenotype of the mutant was related to altered *INO1* gene expression and whether NA might have an effect on *INO1* transcription. Since the *npt1*Δ mutant is able to grow in I- NA+ medium at 30^{o}C, *INO1* expression levels in the *npt1*Δ strain were initially measured and compared to wild type at 30^{o}C.

INO1 expression levels in the *npt1*Δ strain shifted from I+ NA+ to I- NA+ 30^{o}C (experiment 2 in Figure 13) were decreased by 30% compared to the levels of the wild type strain under identical conditions (experiment 1 in Figure 13). However, *INO1* expression levels in *npt1*Δ cells grown in the absence of NA (experiment 4 in Figure 13) were not significantly different from the *INO1* expression levels of the wild type strain under identical conditions (experiment 3 in Figure 13).

Therefore, while *INO1* expression levels in the wild type strain were not significantly affected by the presence or absence of NA (compare experiment 1 to 3 in Figure 13), the *npt1*Δ strain exhibited significantly lower *INO1* expression at 3 hrs after a shift to I- NA+ medium compared with a shift from I+ NA- to I- NA- medium at 30^{o}C (compare experiment 2 to 4 in Figure 13).

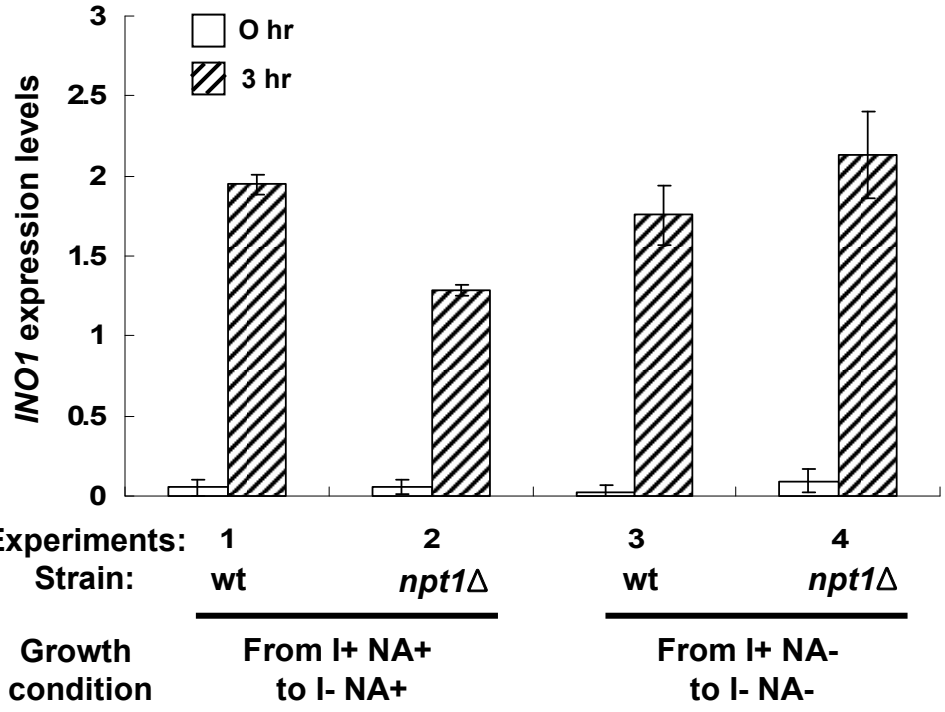


Figure 13. The absence of NA in the growth medium of the *npt1*Δ mutant resulted in *INO1* expression levels to levels equivalent to the wild type at 30°C. Cells were grown in medium containing inositol and NA (I+ NA+) or lacking NA (I+ NA-) until mid-logarithmic phase at 30°C. Cells were then shifted to I- NA+ or I- NA- medium, respectively. *INO1* expression levels were measured at 0 (white bars) and 3 hrs (striped bars) after the shift to new growth conditions. *INO1* mRNA levels were normalized to *ACT1* mRNA levels. Data are averages from three independent experiments, and the error bars represent the standard deviation (S.D), n=3.

INO1 expression levels were also determined at 37°C since the Ino⁻ phenotype of *npt1*Δ is only observed at 37°C. The design of these experiments was identical to that used for the experiment at 30°C shown in Figure 12. At mid-logarithmic phase (A_{600}=0.5-0.6), cells grown in I+ NA+ or I+ NA- medium at 37°C were shifted to I- NA+ or I- NA- medium, respectively and harvested at 0 to 5 hrs.

Overall, *INO1* expression levels at 37°C were lower than the levels observed at 30°C in both wild type and *npt1*Δ strains. At 37°C, *INO1* expression levels in wild type cells were lower in the absence of NA, compared to levels observed in cells grown in the presence of NA. At the higher temperatures, the absence of NA clearly correlated with lower *INO1* expression levels in wild type (Figure 14), whereas the presence or absence of NA had no effect on *INO1* expression levels in the wild type strain at 30°C (Figure 13). However, in contrast to wild type, *INO1* expression levels in *npt1*Δ cells shifted to I- NA- medium increased to a level about 1.8-fold, compared to the levels in *npt1*Δ cells shifted to I- NA+ medium at 3 hrs following media shift (Figure 14).

Compared with wild type cells, in the presence of NA at 37°C, *INO1* expression levels in *npt1*Δ cells were almost 50% lower than the levels observed in wild type at 3 hrs following media shift. However, it is important to point out that the difference between wild type and *npt1*Δ in *INO1* expression in I- NA+ medium had disappeared by 4 and 5 hrs since *INO1* expression levels had dropped in wild type. Thus, it is not clear that the difference in *INO1* expression alone compared to wild type is sufficient to explain the failure of *npt1*Δ cells to grow on I- NA+ at 37°C.

Summary : In the absence of NA, *INO1* expression levels in *npt1*Δ cells were equivalent to those in wild type at 37°C at 3 hrs (Figure 14) , but the removal of NA from the growth medium only partially suppressed the growth defect in *npt1*Δ at 37°C on I- medium (Figure 4). Therefore, the Ino⁻ phenotype of *npt1*Δ cells at 37°C is only partially related to the *INO1* expression levels.

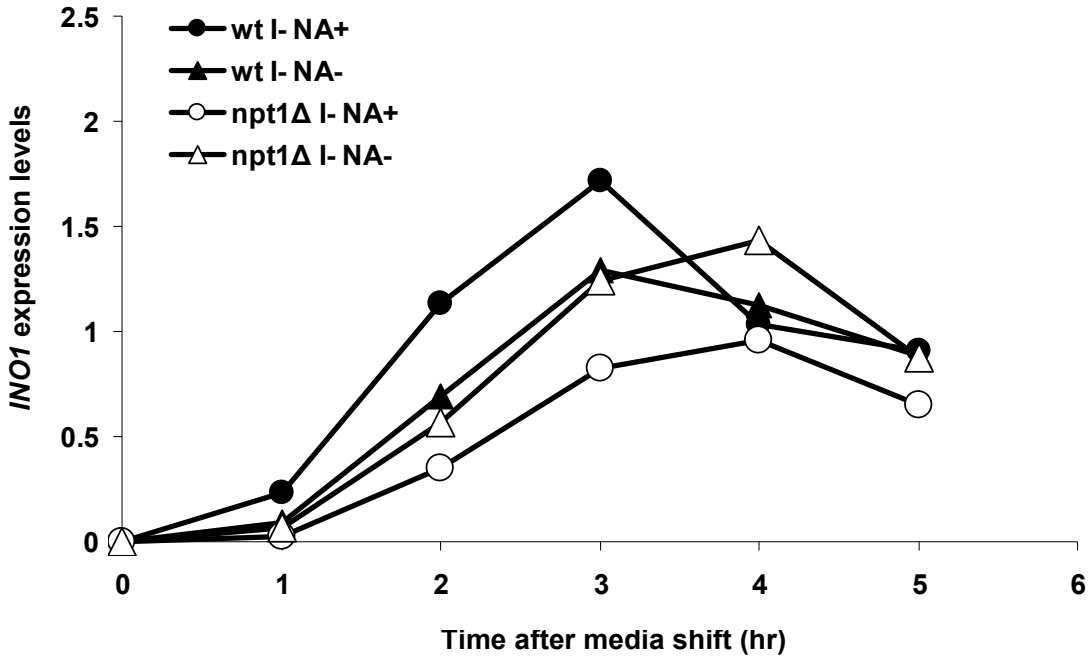


Figure 14. *INO1* expression levels in the *npt1*Δ and wild type strains at 37°C. Cells were grown in I+ NA+ or I+ NA- medium until mid logarithmic phase (A_{600}=0.5-0.6) at 37°C. Cells were then shifted to I- medium keeping NA constant level at same temperature. *INO1* expression levels were measured at 0 to 5 hrs following a shift to the medium lacking inositol. *INO1* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

B. Transcriptional regulation of the genes involved in NAD^+ metabolism in response to inositol and nicotinic acid (NA)

B. 1. Intracellular NAD^+ levels in wild type and *ino1*Δ strains

Ino1p needs NAD^+ for its catalytic activity (Byun and Jenness 1981). Therefore, NAD^+ levels might affect Ino1p activity and thus affect inositol synthesis. It is also possible that elevated Ino1p activity might influence the demand for NAD^+ sufficiently to affect intracellular NAD^+ levels. Therefore, the Ino^- phenotype of *npt1*Δ might due to increased cellular demand for NAD^+ when Ino1p is fully derepressed. It has been reported that growth of yeast cells in medium lacking NA results in decreased intracellular NAD^+ levels compared to cells grown in medium containing NA (Bedalov, Hirao et al. 2003).

Therefore, the effect of growth of wild type cells in the presence or absence of inositol and NA on intracellular NAD^+ levels were tested. The measurement of intracellular NAD^+ levels was accomplished in cells grown continuously in four different media, I+ NA+, I- NA+, I+ NA- and I- NA-, at 30^{o}C. As described in the Materials and Methods, this assay involves converting NAD^+ to NADH by treatment with alcohol dehydrogenase. The levels of NADH are then measured at absorbance 340 nm. The absorbance of each sample was used to calculate the concentration of NAD^+ from NAD^+ standard curve generated using commercial NAD^+ from Sigma. NAD^+ concentrations were used to produce a standard curve, ranging from 0.01 to 0.1 mM.

Interestingly, intracellular NAD^+ levels in wild type did respond to inositol when NA was present. Steady state intracellular NAD^+ levels in wild type cells grown in medium lacking inositol with NA (I- NA+) were found to be higher than in cells grown in medium containing inositol and NA (I+ NA+) (Figure 15). However, the

absence of NA resulted in at least 2-fold decrease NAD^+ levels compared to the levels in wild type cells grown in the presence of NA whether inositol was present or not. Thus, intracellular NAD^+ levels appeared to respond significantly to inositol only when NA was present. Overall, NA appears to be a more critical factor in determining intracellular NAD^+ levels than inositol in wild type cells.

The increased NAD^+ levels observed in wild type cells grown in the presence versus the absence of inositol, when NA is present, could be due to the fact that Ino1p requires NAD^+ as a cofactor for its activity (Maeda and Eisenberg 1980; Mauck, Wong et al. 1980). Therefore, it was investigated whether the presence of Ino1p affected intracellular NAD^+ levels. The *ino1*Δ mutant lacks Ino1p and cannot synthesize inositol *de novo* and therefore, is an inositol auxotroph. For these reasons, the *ino1*Δ mutant cannot be grown continuously in medium lacking inositol. However, the *ino1*Δ strain shows no loss of viability for at least 3 hrs following a shift from medium containing inositol to medium lacking inositol (Henry, Atkinson et al. 1977). To determine NAD^+ levels in the *ino1*Δ strain in I- NA+ medium, cells were grown in I+ NA+ medium to mid-logarithmic phase at 30°C and then shifted to I- NA+ medium. Cells were harvested at 3 hrs following the shift. At this time, cultures had reached density of about A_{600}= 1.0. The wild type strain was tested under identical conditions for comparison. As a further control, intracellular NAD^+ levels following continuous growth in I+ NA+ medium in both wild type and *ino1*Δ cells harvested at A_{600}= 1 were measured.

NAD^+ levels observed in *ino1*Δ cells grown continuously in I+ NA+ medium were similar to the levels observed in the wild type strain (Figure 16). The NAD^+ levels in the *ino1*Δ strain at 3 hrs following the shift from I+ NA+ to I- NA+ medium were higher than the NAD^+ levels detected in *ino1*Δ cells grown continuously in I+ NA+ medium (Figure 16). Intracellular NAD^+ levels in wild type cells continuously

grown in I- NA+ medium were higher than in cells grown in I+ NA+ medium (Figure 15). However, when wild type cells were shifted from I+ NA+ to I- NA+ medium, NAD^+ levels at 3 hrs after the shift were 20% lower than the level in cells grown continuously in I+ NA+ medium (Figure 16). In contrast, the NAD^+ level in *ino1*Δ cells shifted to I- NA+ medium were similar to the NAD^+ levels in wild type cells grown continuously in I- NA+ medium (Figure 16).

These results suggest that growth in the absence of inositol results in higher NAD^+ levels that are, at least in part, due to the impact of Ino1p expression and/or activity. The *ino1*Δ mutant, unlike the wild type strain, did not show a decrease in NAD^+ levels immediately after the shift to I- medium, a condition that results in derepression of Ino1p and a simultaneously decrease in NAD^+ levels in wild type cells. The decreased NAD^+ level seen in wild type at 3 hrs might result from immediate demand for NAD^+ for Ino1p activity required for synthesis of inositol under conditions lacking inositol. However, over longer periods of continuous growth in the absence of inositol, wild type cells apparently adapt to the new environment that demands a higher NAD^+ level as shown in the data from wild type cells grown continuously in I- NA+ medium (Figure 15 and 16).

Summary : NAD^+ levels respond to factors in the external environment, such as availability of inositol and NA. The increased NAD^+ levels in wild type cells grown in I- NA+ medium compared to I+ NA+ medium may be partially due to the impact of Ino1p.

B. 2. Low intracellular NAD^+ levels observed in the *npt1*Δ strain

Mutations in NAD^+ metabolism have been reported to affect intracellular NAD^+ levels. For example, the *npt1*Δ mutant grown in YPD medium was reported to have low NAD^+ levels compared to wild type cells grown in the same medium

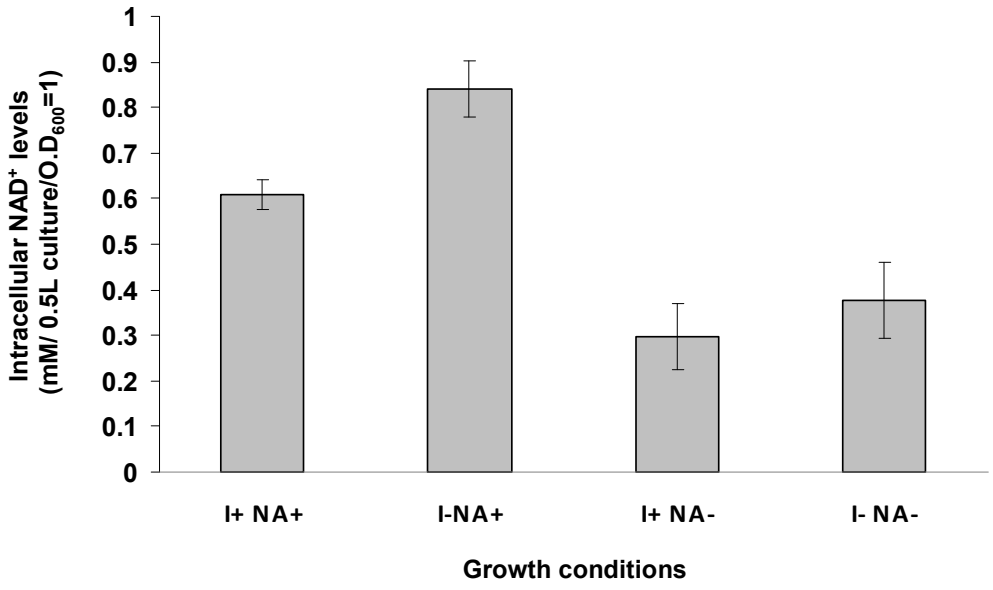


Figure 15. Intracellular NAD^+ levels in the wild type strain grown continuously in several different growth media. Cells were grown in continuously in four different media (i.e. I+ NA+, I- NA+, I+ NA- and I- NA-) at 30^{o}C. Cells were harvested at A_{600}=1.0. NAD^+ levels were measured as described in the Materials and Methods using the alcohol dehydrogenase assay which converts NAD^+ to NADH which is then detected at absorbance 340 nm. Absorbance 340 nm was used to calculate the concentration of NAD^+ using a standard curve as described in the Materials and Methods. Data represent the average of three independent experiments, and the error bars represent the standard deviation (S.D), n=3.

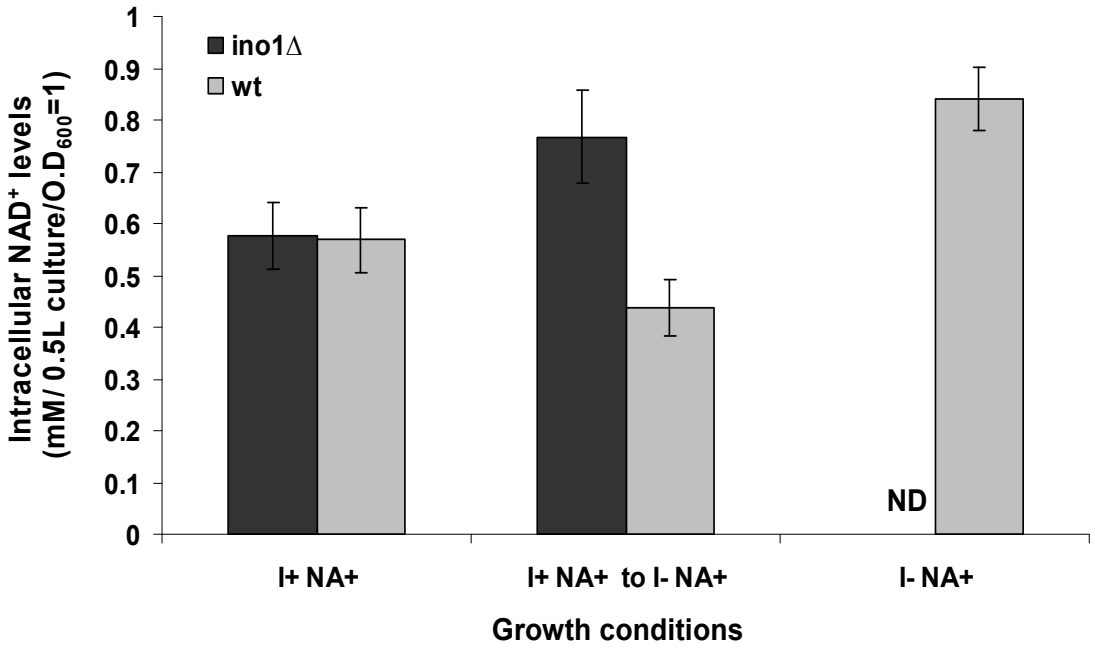


Figure 16. Intracellular NAD^+ levels in the *ino1*Δ strain compared with wild type. Cells were grown in the presence of inositol and NA (I+ NA+) at 30°C. Half of each culture was shifted to absence of inositol with NA (I+ NA+ to I- NA+). The remainder was kept in the original growth medium (I+ NA+). Cells were filtered at 3 hrs after the media shift or when the culture reached at A_{600}=1.0. For comparison, wild type cells were grown continuously in I- NA+ medium until A_{600}=1.0. However, *ino1*Δ cells cannot grow continuously in I- NA+ medium, so under the condition, experiment could not be carried out (marked as ND, not done). NAD^+ levels were measured as described in the Materials and Methods using the alcohol dehydrogenase assay. Data represent the average of three independent experiments, and the error bars represent the standard deviation (S.D), n=3.

(Sandmeier, Celic et al. 2002; Smith, Avalos et al. 2002; Bedalov, Hirao et al. 2003). Since growth of wild type cells in the absence of inositol results in higher NAD^+ levels, It was questioned whether NAD^+ levels in the *npt1*Δ mutant would respond to inositol and/or NA in a similar manner at 30°C. Growth conditions of cells were identical to those in the previously described experiments involving wild type cells grown continuously in I+ NA+, I- NA+, I+ NA- and I- NA- media (Figure 15).

Unlike wild type, the overall NAD^+ levels in the *npt1*Δ mutant did not differ significantly under any of the growth conditions tested, I+ NA+, I- NA+, I+ NA- or I- NA- media at 30°C (Figure 17). The NAD^+ levels observed in *npt1*Δ were almost two-fold lower than the levels observed in wild type cells grown in the presence of NA, but were similar to the levels in wild type cells grown in the absence of NA (Figure 17).

Summary : Inositol and NA had no affect on the NAD^+ levels in the *npt1*Δ mutant, which exhibits consistently low NAD^+ levels.

B. 3. Changes in NAD^+ levels in other mutants involved in NAD^+ metabolism

Mutations in the NAD^+ biosynthetic pathway alter NAD^+ levels. The *hst1*Δ strain exhibits an almost 2-fold increase NAD^+ levels and the *bna2*Δ strain shows a decrease 20% in NAD^+ levels when grown in the absence of tryptophan compared to wild type cells grown under the same conditions. However, NAD^+ levels in sirtuin mutants, with the exception of *hst1*Δ, did not differ from wild type cells (Bedalov, Hirao et al. 2003). The *pnc1*Δ, *bna1*Δ and *qpt1*Δ mutants reportedly have NAD^+ levels similar to wild type grown in YPD medium (Sandmeier, Celic et al. 2002). However, it has been recently reported that NAD^+ levels in the *pnc1*Δ mutant grown in SC medium were lower than the levels in wild type cells grown in the same medium (McClure, Gallo et al. 2008). In these experiments, cells were continuously grown in I+ NA+, I- NA+, I+ NA- and I- NA- media at 30°C.

The *pnc1*Δ strain grown in I+ NA+ and I- NA+ media exhibited no difference in NAD^+ levels compared with the levels in wild type under the same growth conditions. However, in I+ NA- or I- NA- medium, NAD^+ levels in the *pnc1*Δ strain were slightly decreased compared to the levels in wild type (Figure 18.A).

The *hst1*Δ mutant did not exhibit elevated NAD^+ levels in comparison to wild type when grown continuously in any of the four different growth conditions employed in this study (Figure 18.B). Like wild type, *hst1*Δ exhibited low NAD^+ levels in the absence of NA and showed increased NAD^+ levels when supplied with exogenous NA. However, these results differ from the previous report of Bedalov *et al*. (Bedalov, Hirao et al. 2003). They reported that the *hst1*Δ strain maintained the same NAD^+ levels regardless of the presence and absence of NA and/or tryptophan.

When the *NPT1* gene is deleted in the *hst1*Δ mutant, the pattern of NAD^+ levels under the four different growth conditions differed from the pattern observed in the *hst1*Δ strain. In I+ NA+ or I- NA+ medium, the *hst1*Δ*npt1*Δ double mutant strain exhibited lower NAD^+ levels compared to those of the *hst1*Δ and wild type strains. However, NAD^+ levels in *hst1*Δ*npt1*Δ cells grown in I+ NA+ medium were higher than the levels in *npt1*Δ cells, whereas in I- NA+ medium, the NAD^+ levels in *hst1*Δ*npt1*Δ were similar to the levels observed in *npt1*Δ cells. Under the other growth conditions tested (I+ NA- or I- NA- medium), there were no significant differences in the NAD^+ levels in any of the strains tested (Figure 18.B).

Summary : The NAD^+ levels of the *pnc1*Δ and *hst1*Δ strains were similar with the levels in wild type under all conditions tested. The absence of NA is caused to reduce NAD^+ levels in all strains tested, whether inositol was present or not.

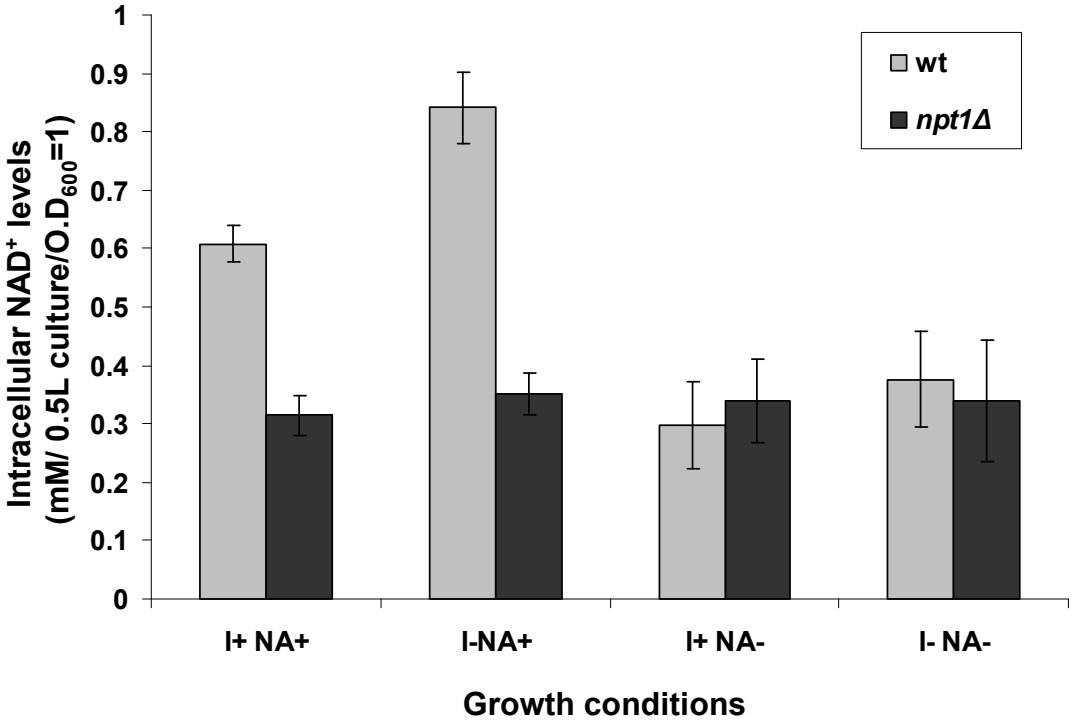


Figure 17. Intracellular NAD^+ levels in the *npt1*Δ mutant compared to wild type cells grown continuously in four different growth conditions. Cells were grown continuously four different growth conditions, presence or absence of inositol and NA, I+ NA+, I- NA+, I+ NA- and I- NA- at 30°C. Cells were harvested at A_{600}=1.0. NAD^+ levels were measured as described in the Materials and Methods using the alcohol dehydrogenase assay which converts NAD^+ to NADH which is then detected at absorbance 340 nm. Absorbance 340 nm was used to calculate the concentration of NAD^+ using a standard curve as described in the Materials and Methods. Data represent the average of three independent experiments, and the error bars represent the standard deviation (S.D), n=3.

A) Intracellular steady-state NAD^+ levels in the *pnc1*Δ strain

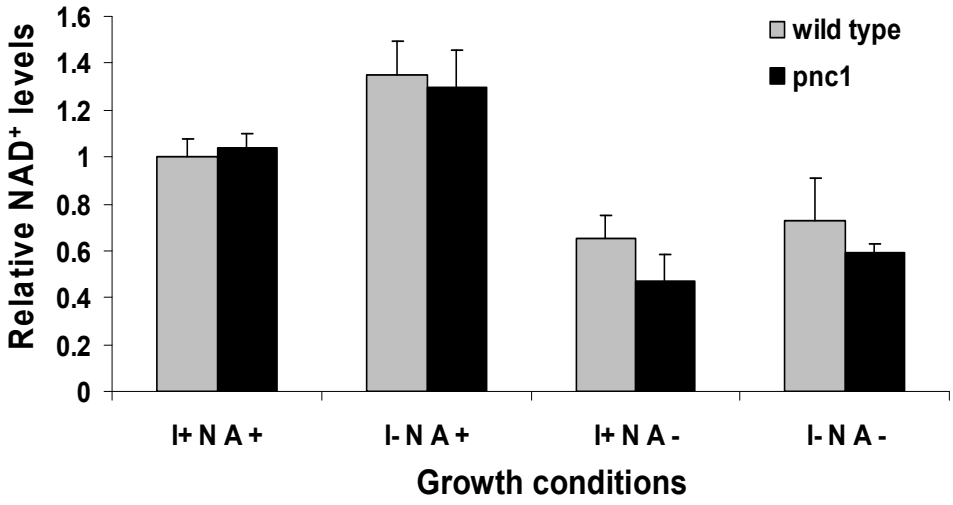


Figure 18.A. Relative intracellular steady-state NAD^+ levels in the *pnc1*Δ mutant involve in NAD^+ metabolism. Cells were grown in four different growth conditions, I+ NA+, I- NA+, I+ NA- and I- NA-. Cells were harvested at A_{600}=1.0. Measurement of NAD^+ levels was followed as described in the Materials and Methods. Relative intracellular steady-state NAD^+ levels in the *pnc1*Δ mutant. Data are the average from three independent experiments, and the error bars represent the standard deviation (S.D).

B) Intracellular NAD^+ levels in the *npt1*Δ, *hst1*Δ, *hst1*Δ*npt1*Δ mutants

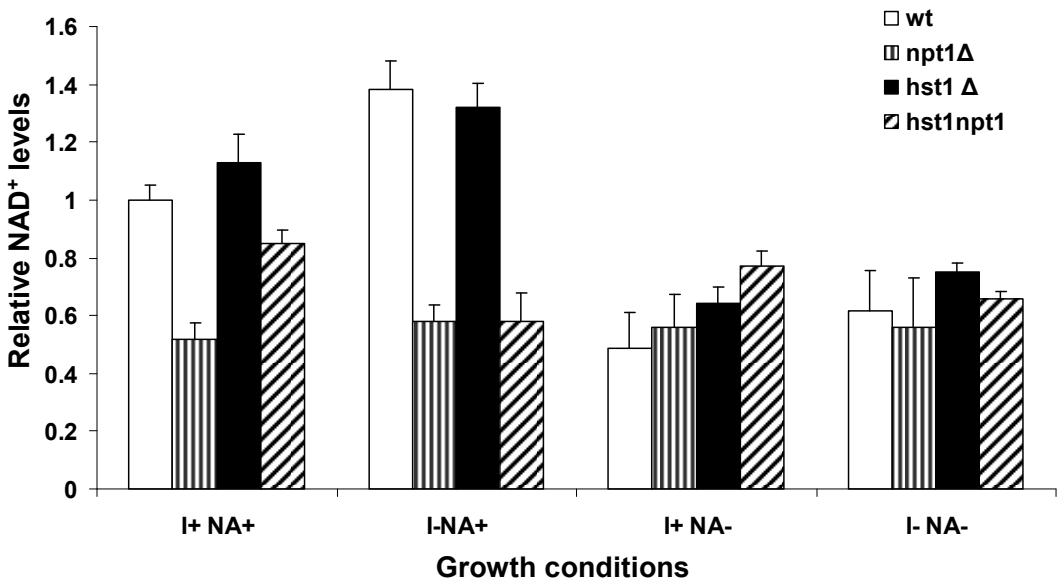


Figure 18.B. Relative intracellular steady-state NAD^+ levels in *hst1*Δ and *hst1*Δ*npt1*Δ strains involve in NAD^+ metabolism. Cells were grown in four different growth conditions, I+ NA+, I- NA+, I+ NA- and I- NA-. Cells were harvested at A_{600}=1.0. Measurement of NAD^+ levels was followed as described in the Materials and Methods. Relative intracellular steady-state NAD^+ levels in the *hst1*Δ and *hst1*Δ*npt1*Δ strains. Data are the average from three independent experiments, and the error bar represents the standard deviation (S.D).

B. 4. Intracellular NAD^+ levels in the *npt1*Δ and wild type strains at 37°C

NAD^+ levels in wild type cells are affected by supplementation with inositol and NA (Figure 15). However, *npt1*Δ cells show low NAD^+ levels under all growth conditions tested (Figure 17). Increasing the growth temperature to 37°C led to decrease *INO1* gene expression in wild type cells (Figure 12). The *npt1*Δ mutant exhibited weak Ino^- phenotype at 37°C (Figure 4) and the Ino^- phenotype was associated with a decrease in *INO1* expression in the *npt1*Δ mutant at 37°C (Figure 14). Therefore, it was questioned whether increased growth temperature would affect NAD^+ levels in *npt1*Δ.

NAD^+ levels in wild type cells grown in I+ NA+ were similar to the levels in I- NA+ medium at 37°C (Figure 19). This result is in contrast to the situation observed at 30°C. At 30°C, NAD^+ levels in wild type cells grown in I- NA+ were increased compared to the levels of cells grown in I+ NA+ medium (Figure 15). The level of NAD^+ in cells grown at 37°C with or without inositol was equivalent to the higher levels observed at 30°C in cells grown in I- NA+ medium. Thus, an increase the growth temperature seems to result in an increased demand for NAD^+ in cells grown in the presence of inositol, while cells grown in the absence of inositol have a higher demand levels at 30°C. However, NAD^+ levels in *npt1*Δ cells grown continuously in I+ NA+ medium at 37°C remained low and were comparable to the levels observed in cells grown in all four growth conditions, I+ NA+, I- NA+, I+ NA- and I- NA- at 30°C (Figure 17 and 20). When *npt1*Δ cells were shifted from I+ NA+ to I- NA+ medium at A_{600}=0.2 and harvested at A_{600}=1.0 at 37°C, no change in NAD^+ level was detected compared with the level observed in other growth conditions (Figure 20). *npt1*Δ cells exhibited low NAD^+ levels under all growth conditions, with little change with respect to different media conditions or temperature (Figure 17 and 20).

Summary : Higher temperatures may result in higher cellular demand for

NAD^+. In wild type cells, NAD^+ levels at 37°C were higher than the levels at 30°C, especially in the presence of inositol. However, in contrast to wild type, the *npt1*Δ mutant exhibited consistently low NAD^+ levels regardless of the presence or absence of inositol and/or NA supplementation or increased growth temperature.

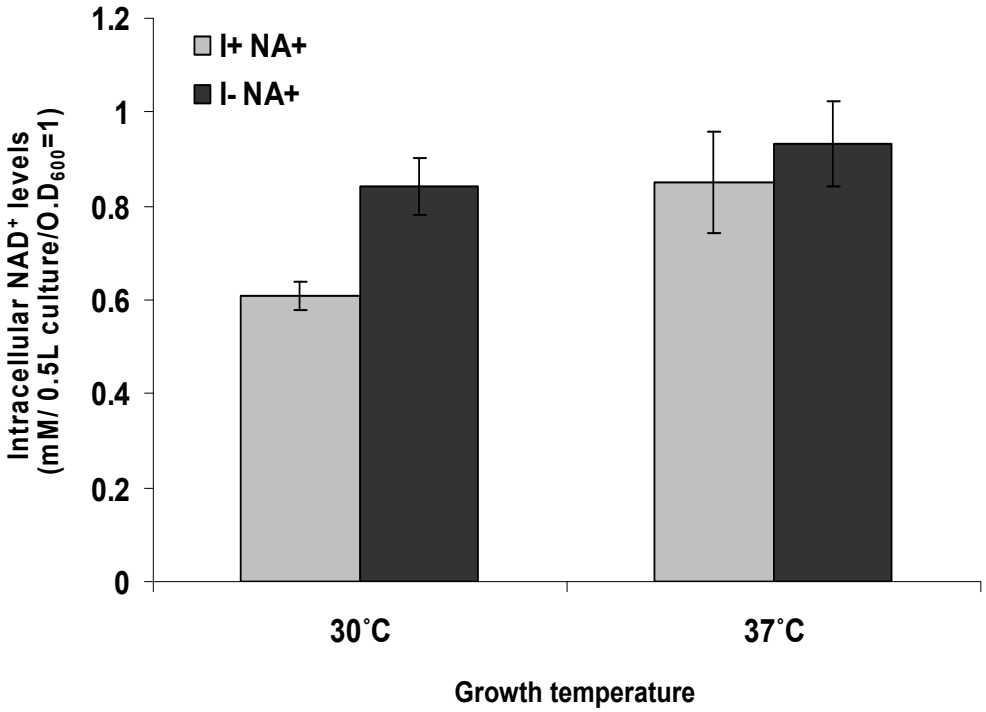


Figure 19. NAD^+ levels in the wild type strain at 37°C compared with 30°C. Cells were grown continuously in I+ NA+ and I- NA+ media at 30 and 37°C. Cells were harvested at A_{600}=1.0. Measurement of NAD^+ levels was accomplished as described in the Materials and Methods. Data are average from three independent experiments, and the error bars represent the standard deviation (S.D).

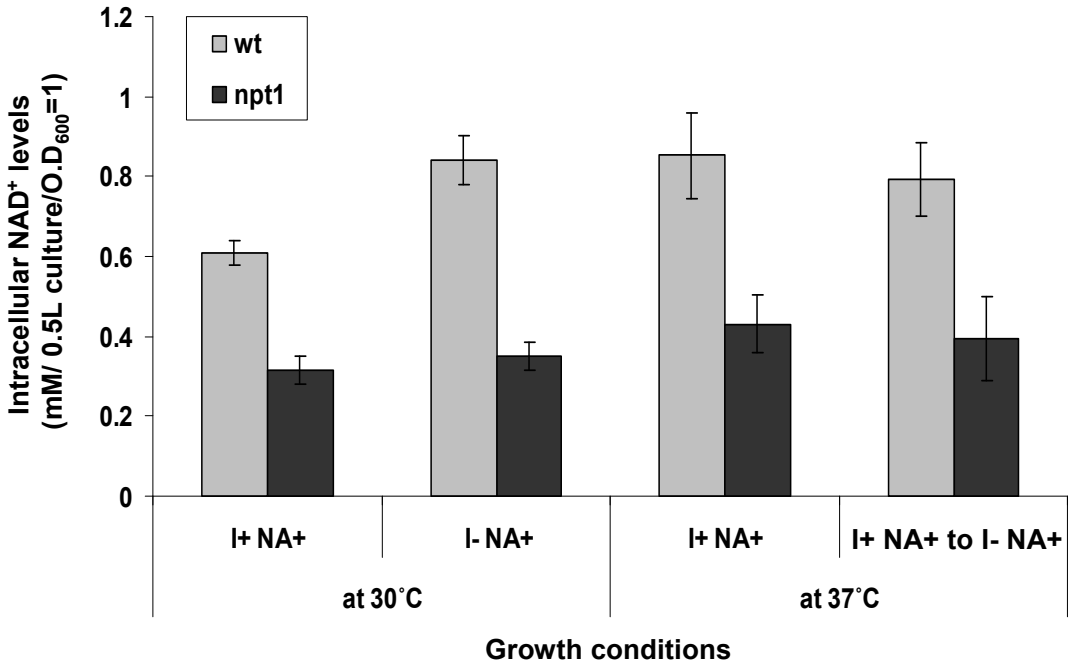


Figure 20. NAD^+ levels in the *npt1*Δ mutant at 30 and 37°C compared with the NAD^+ level in the wild type strain. At 30°C, cells were grown continuously in I+ NA+ and I- NA+ media and then harvested at A_{600}=1.0. At 37°C, these were grown continuously in I+ NA+ medium and then harvested at A_{600}=1.0. However, at 37°C, *npt1*Δ cells will not grow continuously in I- NA+ medium. So, measurement of NAD^+ levels in I- NA+ medium, cells were shifted from I+ NA+ culture to I- NA+ medium at A_{600}=0.2 and then harvested at A_{600}=1.0. Data are average from three independent experiments, and the error bars represent the standard deviation (S.D).

B. 5. Intracellular NA levels in several strains

In the previous section, NAD^+ levels in wild type cells were shown to respond to inositol and NA (Figure 15). However, NAD^+ levels in *npt1*Δ cells remained low under all conditions tested (Figure 17 and 20). The Ino^- phenotype of *npt1*Δ at 37°C was partially suppressed by eliminating NA from the medium (Figure 3 and 4), but no change was observed in the NAD^+ level under these conditions. Also, *INO1* expression levels in *npt1*Δ, in the presence of NA, decreased compared to the levels in wild type at 37°C. Indeed, omitting NA restored *INO1* transcription levels in the *npt1*Δ strain to levels similar to those in wild type at 37°C (Figure 14). Thus, the presence of exogenous NA appears to exert an important effect on phospholipid metabolism in the *npt1*Δ strain, independent of NAD^+ level. The *npt1*Δ mutant is blocked at the step from NA to NaMN (Figure 2.A). Therefore, *npt1*Δ cells might be expected to have abnormal intracellular NA levels in the presence of exogenous NA. It was hypothesized that intracellular NA levels might play a role in regulating the transcription of genes involved in both phospholipid and/or NAD^+ metabolism. Therefore, intracellular NA levels in the *npt1*Δ and wild type strains under various growth conditions were measured. Cells were grown continuously in I+ NA+, I- NA+, I+ NA- and I- NA- media at 30°C and harvested at A_{600}=1.0. NA was extracted from cells using the acid-extraction method described in the Materials and Methods.

NA levels in wild type cells grown in I+ NA+ medium were elevated almost 1.4-fold compared to the levels observed in cells grown in other media, I- NA+, I+ NA- and I- NA- (Figure 21). In contrast to this hypothesis, NA levels in the *npt1*Δ strain did not change much in response to growth conditions. NA levels in *npt1*Δ cells grown in the absence of inositol increased slightly compared to those in cells grown in the presence of inositol, whether NA was present or not (Figure 21). *hst1*Δ cells also showed similar NA levels under all growth conditions tested (Figure 21). In the

absence of inositol (I- NA+ and I- NA- media), *npt1*Δ cells exhibited slightly higher levels of intracellular NA than either wild type or *hst1*Δ. However, in the presence of inositol, NA levels in *npt1*Δ decreased compared to the levels in wild type.

Summary : Intracellular NA levels of all strains tested were consistently maintained regardless of growth conditions.

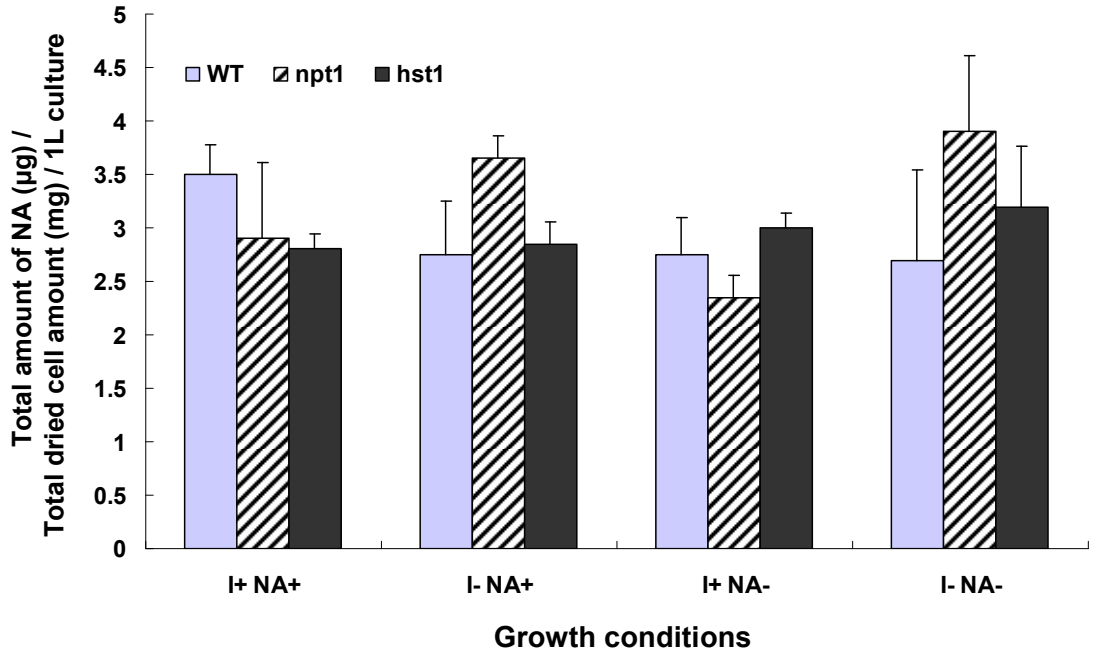


Figure 21. Intracellular NA levels in several strain under four different growth conditions. Cells were grown continuously in the presence or absence of inositol and NA (i.e., I+ NA+, I- NA+, I+ NA- and I- NA- media) at 30^{o}C. Cells were harvested at A_{600}=1.0. NA extraction was accomplished as described in the Materials and Methods. The total amount of NA was calculated by total protein amount in 1L of culture. Intracellular steady-state NA levels in wild type (gray bars) were compared with the *npt1*Δ (striped bars) and *hst1*Δ (black bars) strains**.** Data are averages and error bars represent the standard deviation (S.D).

B. 6. NA excreted from wild type, *pnc1*Δ, and *npt1*Δ cells is capable of supplying the growth of the *bna2*Δ mutant in the absence of NA

Since *npt1*Δ cells are blocked in the step from NA to NaMN (Figure 2. A), it is reasonable to expect that NA might build up in *npt1*Δ cells as a consequence of ongoing NAD^+ metabolism. NA levels in *npt1*Δ cells were somewhat higher than levels in wild type cells grown in the absence of inositol whether NA was present or not (Figure 21). However, the difference in NA levels in *npt1*Δ cells grown under these two conditions did not appear significant. Also, NA levels in *npt1*Δ did not differ very much in any of the conditions tested (Figure 21). However, it is possible that cells can balance NA concentration by exporting NA to the medium and/or by controlling production of NA via NAD^+ metabolism.

To test whether cells exported NA to medium, the *bna2*Δ strain was sprayed on plates lacking NA, on which strains to be tested had been pre-grown for 2 days. Since the *bna2*Δ strain is an NA auxotroph (Kucharczyk, Zagulski et al. 1998), it cannot grow in the absence of NA and any growth detected would necessarily have to be the result of NA exported from the strains pre-grown on the plates. This test is similar in concept to the bioassay used in our laboratory to detect inositol excreted by strains with the overproduction of inositol (Opi^-) phenotype (Greenberg, Reiner et al. 1982).

The plates containing medium lacking NA were pre-cut into four parts, so as to prevent diffusion of NA excreted by the strains being tested. As seen in the control segment of each plate marked as empty, the *bna2*Δ strain could not grow on the medium lacking NA, in the absence of a pre-grown colony of any strain (Figure 22). Unexpectedly, the *bna2*Δ strain grew not only around the *npt1*Δ strain, but also around the wild type and *pnc1*Δ strains (Figure 22). Thus, *npt1*Δ cells released NA out of cells as expected, and the wild type and *pnc1*Δ strains also supported *bna2*Δ growth. The wild type is also presumably releasing NA; but given the block from NAM to NA in

the *pnc1*Δ mutant (Figure 2.A), it seems unlikely that *pnc1*Δ is releasing NA. Rather, *pnc1*Δ probably releases NAM, which can also be used by yeast as a precursor in NAD^+ production. Excretion of NA or NAM using the bioassay was detected in all three strains at 30 and 37°C whether inositol was present or not (Figure 22).

Thus, it appears that cells may maintain intracellular NA concentrations, at least in part, by excretion of NA that is produced in the course of NAD^+ metabolism.

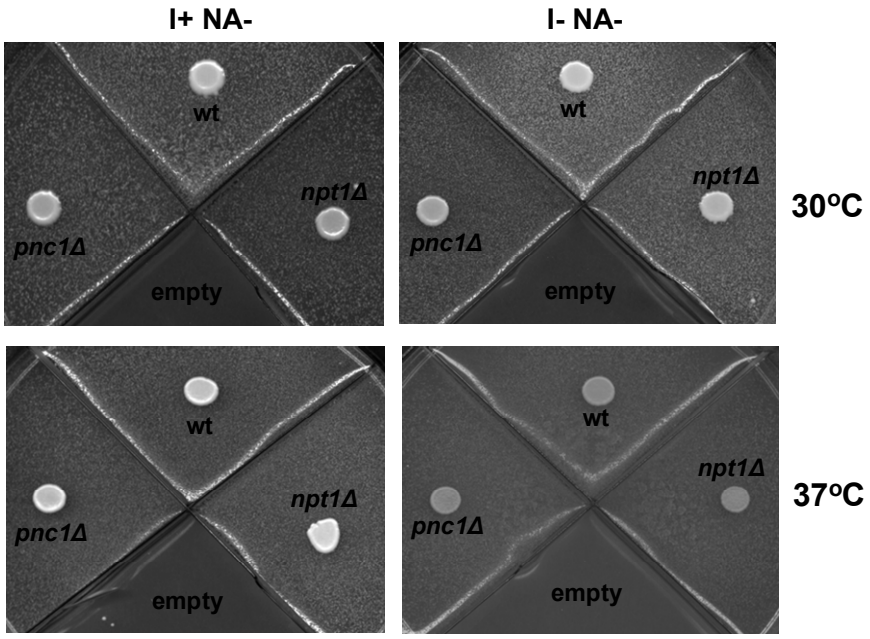


Figure 22. Excreted NA from strains rescued the defect in growth of the *bna2*Δ in the absence of NA. Cells were grown in I+ NA+ or I+ NA- medium at 30°C overnight. Each test plate was cut into four parts. The cells (1.0 A_{600}/ml) were spotted on the plates lacking NA with or without inositol (I+ NA- or I- NA-) and the plates were incubated for 2 days at 30 and 37°C, and the *bna2*Δ strain (shown as white spot on the plates) was then sprayed on the plates, after the strains tested had grown. The plates were then incubated an additional day at 30 or 37°C. Strains used were *npt1*Δ and *pnc1*Δ (testers) and wild type (control). Part of each plate was left empty of any tester strain as a control for the growth of the *bna2*Δ mutant in the absence of NA.

B. 7. *BNA2* transcription in wild type cells is regulated by both inositol and NA

Intracellular NA levels in wild type and *npt1*Δ cells were not much affected by supplementation with either inositol or NA (Figure 21). However, the presence of exogenous inositol and NA clearly affects NAD^+ levels in wild type cells (Figure 15). In wild type cells, regulation of both *TNA1* and *BNA* genes of the *de novo* NAD^+ pathway occurs in response to external NA described in the Introduction. The *npt1*Δ strain relies entirely in the *de novo* pathway since it lacks the salvage pathway due to a block in the critical step from NA to NaMN (Figure 2.A). In the *npt1*Δ strain, to satisfy the cellular demand for NAD^+ in response to changing growth conditions, the *de novo* NAD^+ pathway must be upregulated. The *bna2*Δ mutant, which is blocked in *de novo* NAD^+ synthesis, must derive the NAD^+ from exogenously supplied NA in the reaction catalyzed by Npt1p in the salvage pathway (Figure 2.A). Consistent with this logic, the *bna2*Δ mutant exhibits NA auxotrophy (Kucharczyk, Zagulski et al. 1998) and the *bna2*Δ *npt1*Δ double mutant is not viable (Panozzo, Nawara et al. 2002). The *BNA2* gene is also known to be negatively regulated by Hst1p, an NAD^+-dependent protein deacetylase involved in NAD^+ salvage pathway (Bedalov, Hirao et al. 2003). Thus, the *hst1*Δ mutant was reported to have higher *BNA2* expression levels in SC medium containing 3.25 μM NA and 10 mM inositol by Bedalov *et al*. Interestingly, *BNA2* gene expression was also found to be repressed by addition of inositol in a microarray experiment performed in our laboratory (Jesch, Liu et al. 2006). It was hypothesized that *BNA2* expression responds to NAD^+ levels or to changes in a compound produced by NAD^+ metabolism through Hst1p activity. To test this hypothesis, it was examined whether *BNA2* gene expression responds to inositol and NA supplementation in several strains.

Cells were grown in the presence of inositol with or without NA (i.e., I+ NA+ or I+ NA- medium) at 30°C. At mid-logarithmic phase (A_{600}=0.5-0.6), half of each

culture was shifted to new growth medium lacking inositol but maintaining a constant NA level (i.e., from I+ NA+ to I- NA+ or from I+ NA- to I- NA- medium, respectively). The remainder of each culture was allowed to grow continuously in its original medium. Cells were sampled over several time intervals following a medium shift. Cells grown under continuous growth conditions containing inositol were also harvested at equivalent representative intervals after mid-logarithmic phase.

In the wild type strain, despite generally low levels of expression, *BNA2* levels in cells grown to mid-logarithmic phase in I+ NA- medium were approximately 2-fold higher than in cells grown in I+ NA+ medium (see t=0 in Figure 23). Significantly, cells shifted from I+ NA- to I- NA- medium exhibited a much more dramatic increase in *BNA2* expression than the cells shifted from I+ NA+ to I- NA+. Within 2 to 3 hrs following the shift, *BNA2* expression in cells shifted to I- NA- increased more than 3-fold compared to the levels in cells shifted to I- NA+ medium (Figure 23). However, the level of *BNA2* expression subsequently dropped by 5 hrs by following the shift, returning to levels approaching those seen in wild type cells grown continuously in I+ NA+ medium.

Summary : *BNA2* expression levels in wild type cells grown in the presence of NA were low regardless of the presence of inositol. In contrast, in the absence of NA, when cells were shifted to medium lacking inositol, *BNA2* expression levels increased dramatically but transiently. Thus, *BNA2* expression in wild type cells responds to the removal of both exogenous inositol and NA (Figure 23).

B. 8. *BNA2* expression levels in the *ino1*Δ mutant was similar to wild type

BNA2 expression levels in wild type cells increases in response to both inositol and NA removal (Figure 23). Therefore, it was hypothesized that increased NAD^+ levels might be generated in the absence of inositol due to demand for NAD^+ required

for the activity of Ino1p. Consistent with this idea, wild type cells exhibited a decrease NAD^+ level at 3 hrs following the shift from I+ NA+ to I- NA+ medium (Figure 16). Also, consistent with this hypothesis, the *ino1*Δ mutant, which lacks Ino1p, exhibited higher NAD^+ levels under these same growth conditions (Figure 16). Therefore, *BNA2* expression levels in *ino1*Δ cells was tested using a protocol similar to that used to examine *BNA2* expression in wild type. The only change in the design of the experiments relates the length of time over which samples were collected after the shift to medium lacking inositol. Cells were collected from 0 to 3 hrs since *ino1*Δ cells are known to remain viable only for about 3-4 hrs following a shift to I- medium (Henry, Atkinson et al. 1977).

BNA2 expression levels in the *ino1*Δ mutant responded to NA as shown in initial time (t=0). Prior to the shift to I- medium (t=0), as in wild type cells, *BNA2* expression levels in *ino1*Δ cells grown in the absence of NA were around 2-fold higher than in the same cells grown in the presence of NA (Figure 24). Very little change in *BNA2* expression level was observed in *ino1*Δ cells when they were shifted from I+ NA+ to I- NA+ medium (Figure 24). However, *BNA2* expression levels in *ino1*Δ cells shifted from I+ NA- to I- NA- rose about 2-fold by 2 hrs, in a manner similar to the pattern seen in wild type cells, in which *BNA2* expression levels increased to 3.5-fold by 3 hrs (Figure 23 and 24).

Summary : Wild type and *ino1*Δ cells deprived of inositol and NA exhibited increased *BNA2* expression levels, but the increase was more dramatic in wild type. Therefore, the demand for NAD^+ by Ino1p may play a partial role, at least transiently, in the derepression of the *BNA2* gene as wild type cells are shifted to I- NA-. However, since *BNA2* expression also rose in *ino1*Δ cells, the requirement for NAD^+ by Ino1p does not fully explain the derepression of *BNA2* in wild type cells shifted to I- NA-.

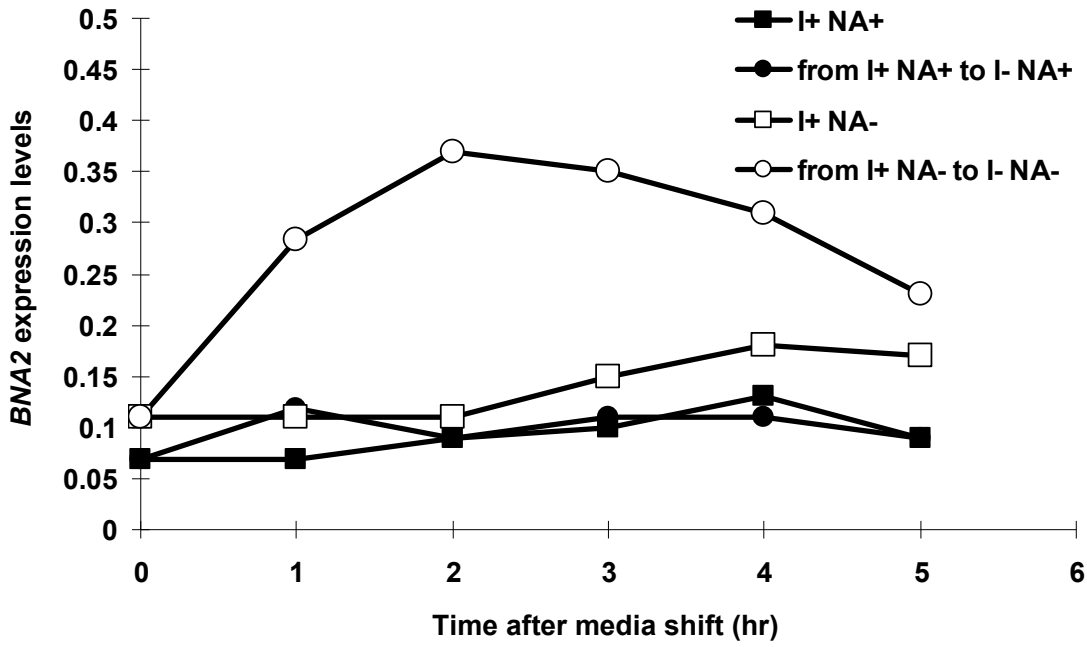


Figure 23. *BNA2* gene expression patterns in the wild type strain following a shift to media lacking inositol at 30°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA-) at 30°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol maintaining a constant NA level (I- NA+ or I- NA- medium, respectively). The remainder of each culture was allowed to continue growing its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data is representative of three experiments.

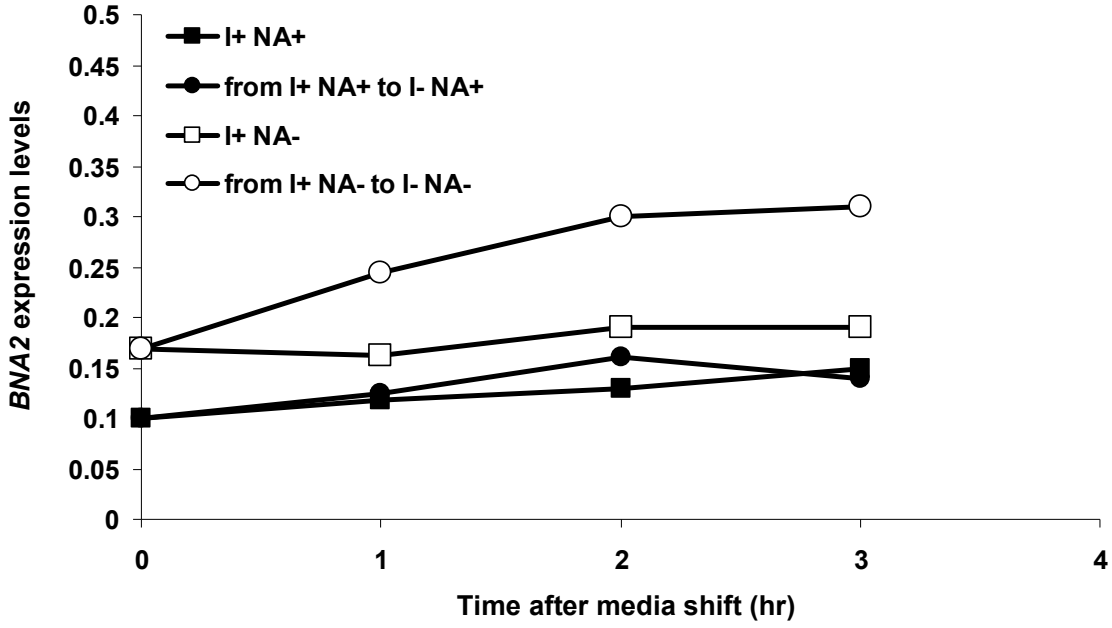


Figure 24. *BNA2* gene expression patterns in the *ino1*Δ strain following a shift to media lacking inositol at 30°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA-) at 30°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol maintaining a constant NA level (I- NA+ or I- NA-, respectively). The remainder of each culture was grown in allowed to continue growing in its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. This data was obtained from one experiment.

B. 9. The *npt1*Δ strain exhibits elevated *BNA2* expression when shifted to medium lacking inositol and containing NA

The *npt1*Δ mutant exhibited constantly low NAD^+ levels (Figure 17 and 20) and intracellular NA levels that did not change (Figure 21) under any of the growth conditions tested; with or without inositol and/or NA supplementation or different growth temperatures, 30 and 37°C. However, as discussed previously, the *npt1*Δ mutant exhibits an Ino^- phenotype at 37°C in I- NA+ medium and this phenotype was partially suppressed when NA was omitted from the growth medium (Figure 3 and 4). These observations suggest that although NA cannot be incorporated via the salvage pathway in *npt1*Δ cells (Figure 2.A), exogenous NA must in some fashion be influencing NAD^+ metabolism. Therefore, I examined *BNA2* expression level to determine whether NA and/or inositol could be influencing NAD^+ metabolism in the *npt1*Δ mutant. The design of these experiments was identical to the approach used for testing the wild type strain at 30°C.

In *npt1*Δ cells allowed to grow continuously in I+ NA+ or I+ NA- medium, *BNA2* expression levels remained constant at the initial levels (Figure 25). However, the patterns were quite different in cells shifted from I+ NA+ to I- NA+ versus cells shifted from I+ NA- to I- NA- (Figure 25). In cells shifted to I- NA- medium, the level of *BNA2* expression initially rose and remained high over 3 hrs and then gradually decreased. In contrast, in cells shifted to I- NA+, levels remained at the initial level seen in *npt1*Δ cells grown in I+ NA+ for 2 hrs and then began to rise, increasing at least 2-fold by 5 hrs, reaching levels as high as those seen only transiently in wild type shifted to I- NA- medium. Thus, in comparison to wild type (Figure 23), the *npt1*Δ mutant exhibited an increase *BNA2* expression levels in cells shifted to I- NA+, as well as, in cells shifted to I- NA- medium (Figure 25).

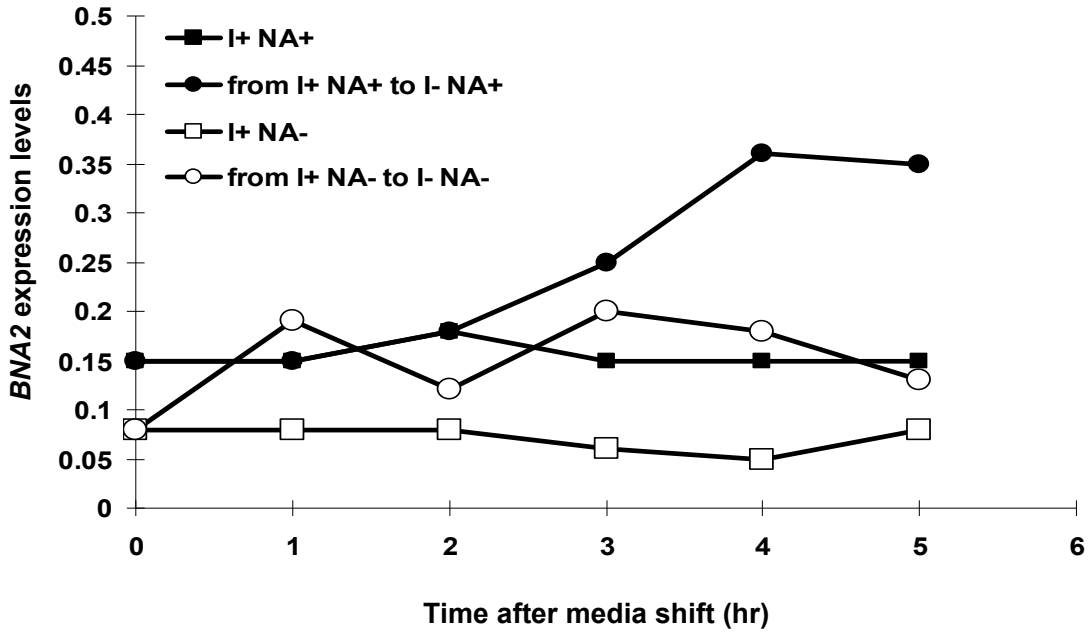


Figure 25. *BNA2* gene expression patterns in the *npt1*Δ strain following a shift to medium lacking inositol at 30°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA- medium) at 30°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol maintaining a constant NA level (I- NA+ or I- NA- medium, respectively). The remainder of each culture was grown in allowed to continue growing its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data is representative at least two experiments.

The interval up to 3 hrs following the shift to medium lacking inositol appears to be a critical point for analyzing the transcription pattern of the specific genes reported here. For example, *INO1* expression levels peaked at this point in wild type cells (Figure 10). Therefore, focus on *BNA2* expression levels at 3 hrs following the shift to medium lacking inositol or the equivalent time in the original media was for comparison and performed at least three or more independent repetitions. At 3 hrs, in cells continuously in the original culture, I+ NA+ or I+ NA- media, *BNA2* expression levels with the *npt1*Δ and wild type strains were similar (Figure 26. A and B). However, when cells were transferred from I+ NA+ to I- NA+ medium, *BNA2* expression levels in *npt1*Δ cells at 3 hrs were 2-fold higher than the levels in wild type cells (Figure 26. A). In contrast, when cells were shifted from I+ NA- to I- NA- medium, *BNA2* expression levels in *npt1*Δ cells at 3 hrs were similar to wild type (Figure 26. B).

Summary : Wild type cells grown continously in I- NA+ medium appear to need more NAD^+ as evidenced by their induction of high levels of *BNA2* expression. However, upon shift to I- NA+ medium, wild type cells do not activate expression of the *BNA2* gene required for *de novo* NAD^+ synthesis, presumably because NA supplied in the growth medium is sufficient to enable to synthesis of NAD^+ via the salvage pathway to meet the increased demand for NAD^+. However, in the absence of both NA and inositol, wild type cells activate the expression of the *BNA2* gene involved in *de novo* NAD^+ synthesis, presumably in order to support increased demand for NAD^+. However, *npt1*Δ cells shifted to medium lacking inositol exhibited increased *BNA2* expression levels whether NA was present or not. This increased expression occurs despite the presence of NA, presumably because *npt1*Δ cells cannot utilize NA as a precursor of NAD^+ synthesis. Furthermore, the presence of exogenous NA in the growth medium of the *npt1*Δ strain seems to increase the energy

requirement of the strain resulting in higher *BNA2* expression, perhaps because of the need to balance intracellular NA levels by exporting NA produced from NAD^+ metabolism against a high exogenous concentration. Thus, removal of NA from growth medium of *npt1*Δ cells is a critical factor in restoring wild type levels in *BNA2* and *INO1* expression levels when the cells are shifted from medium containing inositol to medium lacking inositol (Figure 14 and 26).

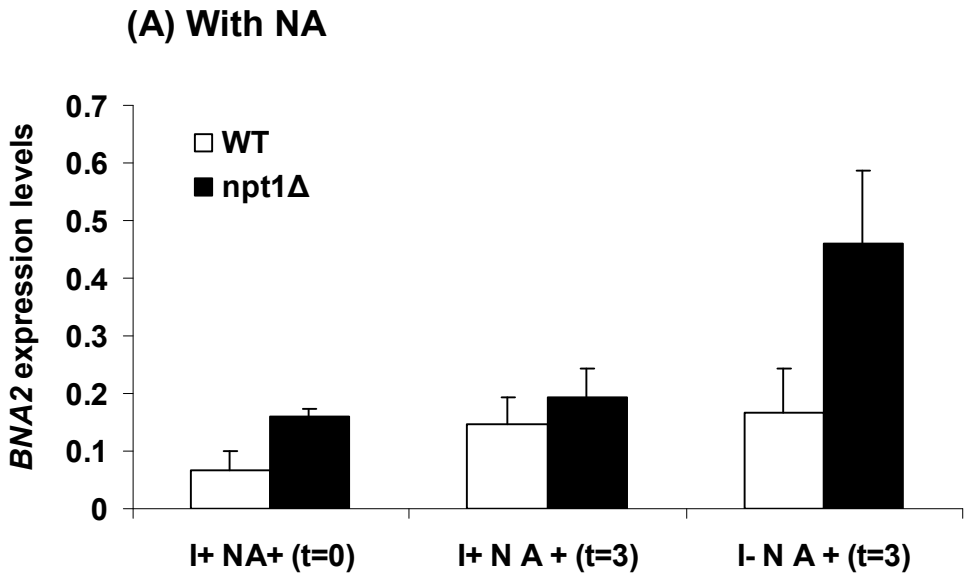


Figure 26. A. *BNA2* gene expression levels at 3 hrs following the medium shift to I- medium with NA (A) in *npt1*Δ and wild type. At A_{600}=0.5-0.6 (t=0) in I+ NA+ medium, half of each culture was shifted to the medium lacking inositol with NA (I- NA+). The remainder of each culture was continuously grown in the original I+ NA+ medium. Samples were harvested at the time of the shift (t=0) or 3 hrs (t=3) after the media shift or an equivalent time in the original growth medium. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data represents the average of three independent experiments, and error bars represent the standard deviation (S.D), n=3.

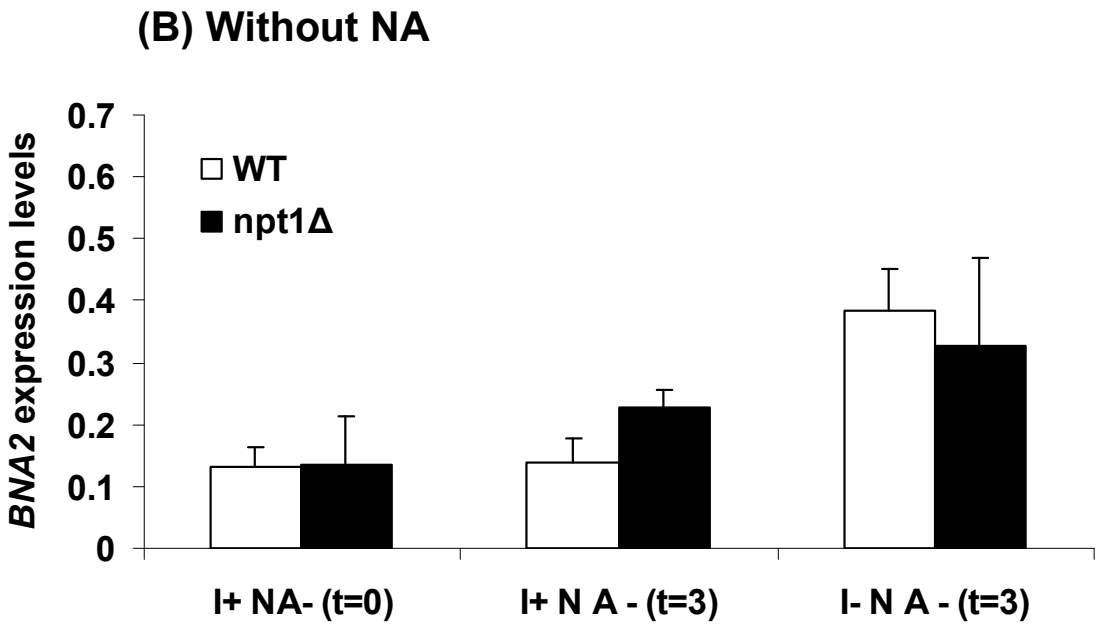


Figure 26. B. *BNA2* expression levels at 3 hrs following the medium shift to I- medium without NA (B) in *npt1*Δ and wild type. Cells were grown in the presence of inositol without NA (I+ NA-) at 30°C. At A_{600}=0.5-0.6 (t=0), half of each culture was shifted to the medium lacking inositol without NA (I- NA-). The remainder of each culture was continuously grown in the original I+ NA- medium. Samples were harvested at the time of the shift (t=0) or 3 hrs (t=3) after the media shift or an equivalent time in the original growth medium. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data represents the average of three independent experiments, and error bars represent the standard deviation (S.D), n=3.

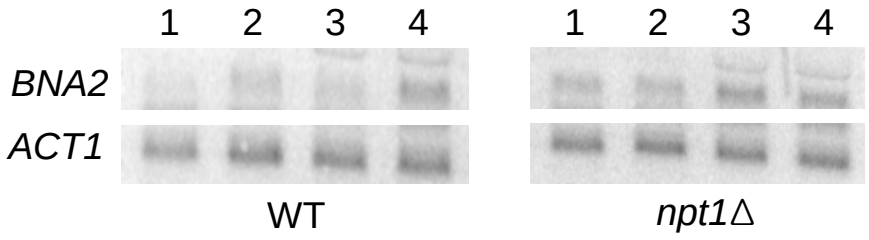


Figure 26. C. Representative Northern blot showing induction of *BNA2* in wild type and *npt1*Δ cells following a shift to medium lacking inositol. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA- medium) at 30^{o}C. At A_{600}=0.5-0.6, half of each culture was shifted to the medium lacking inositol without NA (I- NA+ or I- NA- medium). The remainder of each culture was continuously grown in the original I+ NA+ or I+ NA- medium. Samples were harvested at 3 hrs (t=3) after the media shift or an equivalent time in the original growth medium. *BNA2* transcript abundance was analyzed by Northern blotting in both wild type and *npt1*Δ cells. *ACT1* transcript levels served as loading control. These blot results are quantified as shown in Figure 26. A and B. Lane 1; I+ NA+ (t=3) in Figure 26. A., lane 2; I+ NA- (t=3) in Figure 26. B., lane 3; I- NA+ (t=3) in Figure 26. A., lane 4; I- NA- (t=3) in Figure 26. B.

B. 10. *BNA2* gene expression levels in the *hst1*Δ strain increased after a shift to I- medium whether NA was present or not at 30°C

Hst1p, an NAD^+-dependent protein deacetylase, is believed to sense and regulate NAD^+ levels by causing repression of expression of the *BNA* genes and the *TNA1* gene. Deletion of *HST1* has been reported to result in upregulation of the *BNA2* gene even in the presence of NA (Bedalov, Hirao et al. 2003). In these experiments, *BNA2* transcription in wild type cells was regulated in response to in the absence of both inositol and NA (Figure 23). Therefore, I tested whether Hst1p was involved in the transcriptional regulation of the *BNA2* gene in response to exogenous inositol and/or NA. *BNA2* expression in the *hst1*Δ strain was examined at 30°C in experiments identical in design to the studies on wild type and *npt1*Δ as shown in Figure 23 and 25.

At the initial time point (t=0), *hst1*Δ cells grown in the absence of NA exhibited slightly higher *BNA2* expression levels than the same cells grown in the presence of NA. When cells were continuously grown in medium containing inositol, *BNA2* expression levels remained constant whether NA was present or not (Figure 27). However, when *hst1*Δ cells were shifted to medium lacking inositol, I- NA+ or I- NA-, *BNA2* expression levels increased dramatically under both growth conditions within 3 hrs following the media shift. After 3 hrs, *BNA2* expression levels in *hst1*Δ cells shifted to I- NA+ medium decreased rapidly, while levels in cells shifted to I- NA- medium decreased more gradually (Figure 27). Overall *BNA2* expression levels in *hst1*Δ cells at 30°C under all growth conditions tested were higher than the levels in wild type and *npt1*Δ strains at 30°C (compare Figure 27 with Figure 23 and 25).

Summary : The levels of *BNA2* expression in *hst1*Δ increased after the shift to I- medium whether NA was present or not, like the *npt1*Δ strain. However, the *BNA2* expression levels in the *hst1*Δ strain were higher than the levels in *npt1*Δ cells.

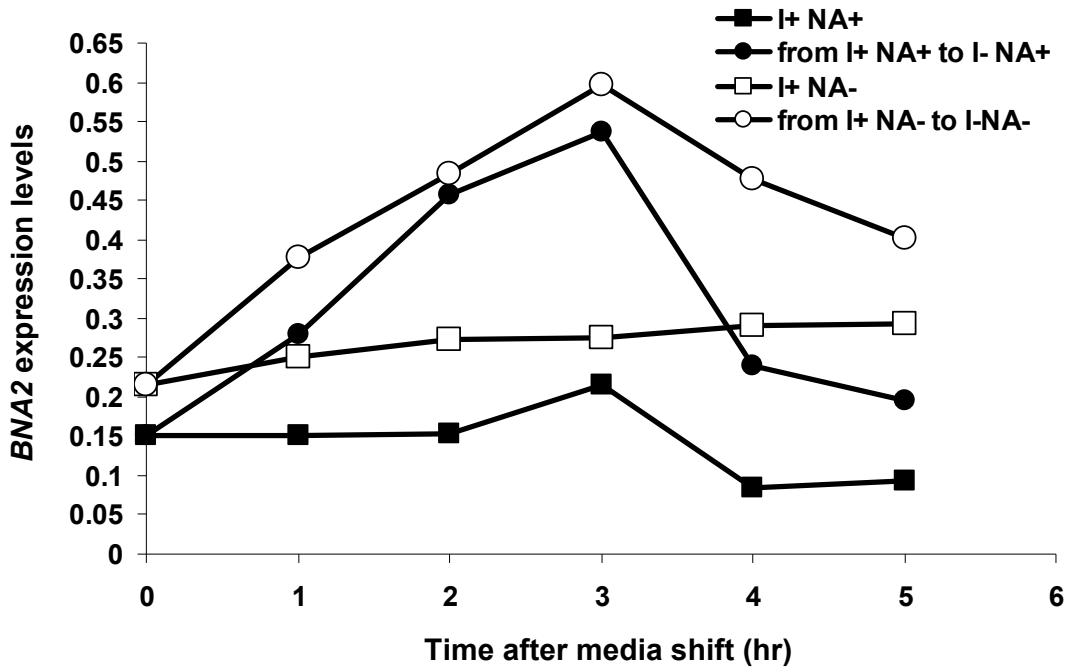


Figure 27. *BNA2* gene expression patterns in the *hst1*Δ strain following a shift to medium lacking inositol at 30°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA- medium) at 30°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol maintaining NA levels constant (I- NA+ or I- NA-, respectively). The remainder of each culture was grown in allowed to continue growing in its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

B. 11. Growth at 37°C results in increased *BNA2* expression in the wild type and *npt1*Δ strains

High temperature (37°C) is a factor associated with changes the patterns of gene expression, as discussed in the previous section of this thesis. For example, *INO1* expression levels in wild type and *npt1*Δ cells at 37°C decreased compared to those at 30°C (Figure 13 and 14). Therefore, it was questioned whether higher temperatures might affect *BNA2* expression level since NAD^+ levels in wild type cells increased at 37°C (Figure 19). These experiments were carried out using a design identical to that used to measure *BNA2* expression level at 30°C (Figure 23). Cells were grown in I+ NA+ or I+ NA- medium at 37°C to mid-logarithmic phase (t=0). Half of each culture was shifted to medium lacking inositol, i.e., to I- NA+ or I- NA- medium. The remainder of each culture was continued in the original growth medium, I+ NA+ or I+ NA- medium.

At the initial time point (t=0), prior to the media shift, wild type cells grown I+ medium exhibited slightly higher *BNA2* expression levels in the absence of NA compared to the levels in cells grown in the presence of NA. *BNA2* expression levels of cells grown continuously in medium containing inositol maintained the low initial expression levels (Figure 28). However, when wild type cells were shifted from I+ NA+ to I- NA+ medium at 37°C, *BNA2* expression levels increased, peaking at 3 hrs and then gradually decreasing (Figure 28). The levels at 37°C were 2-fold higher than the levels at 30°C, especially at 2 and 3 hrs. When wild type cells were shifted from I+ NA- to I- NA- at 37°C, *BNA2* expression levels increased dramatically to a level around 3-fold higher than the initial level and the level remained high throughout the experiment (Figure 28). However, dramatically increased in *BNA2* expression levels in wild type cells shifted to I- NA- medium were observed at 30 and 37°C, with similar high levels at both temperatures (compare Figure 23 with Figure 28).

Interestingly, the *BNA2* expression pattern in *npt1*Δ cells at 37°C responded to inositol whether NA was present or not (Figure 29). At the initial time point (t=0), *BNA2* transcript levels were virtually identical under all growth conditions. However, when *npt1*Δ cells were shifted to media lacking inositol (I- NA+ or I- NA- media), *BNA2* expression levels increased over time, regardless of the presence of NA (Figure 29). *BNA2* expression levels in *npt1*Δ cells shifted to medium lacking inositol were around 2-fold higher at 37°C than the levels at 30°C (Figure 25 and 29).

The overall *BNA2* expression levels in the *npt1*Δ mutant under all growth conditions tested were higher than wild type levels at 37°C (Figure 28 and 29). At 37°C, *BNA2* expression levels in *npt1*Δ cells shifted from I+ NA+ to I- NA+ medium increased 2-fold compared to the levels in wild type in the same growth condition. In contrast, *BNA2* expression levels in *npt1*Δ cells shifted from I+ NA- to I- NA- medium at 37°C were similar to the levels seen in wild type cells grown under the same growth conditions (Figure 28 and 29). Thus, similar to the pattern observed at 30°C, when cells were shifted to I- medium at 37°C, removal of NA resulted in similar increases in *BNA2* expression level in wild type and *npt1*Δ cells.

Summary : Increased growth temperatures resulted in higher *BNA2* expression levels in both wild type and *npt1*Δ than the levels observed at 30°C. The effect of temperature was most pronounced in cells shifted to medium lacking inositol. *BNA2* expression levels in wild type cells seem to respond more to NA removal than increased growth temperatures. In contrast, *BNA2* expression in the *npt1*Δ strain responded more dramatically to the removal of inositol at higher growth temperatures than to the absence of NA.

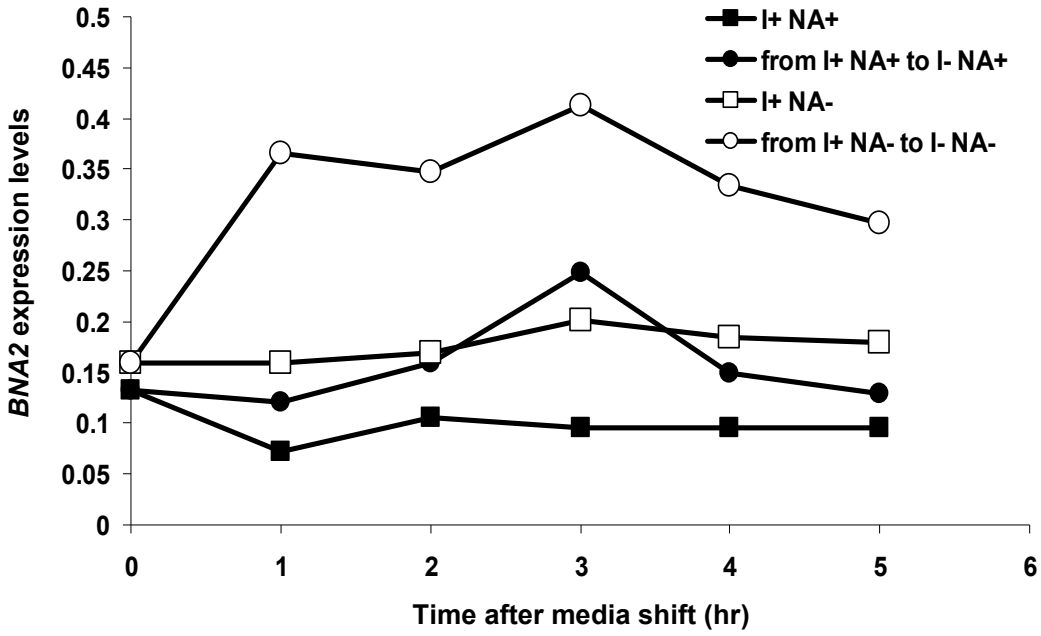


Figure 28. *BNA2* gene expression patterns in the wild type strain following a shift to medium lacking inositol at 37°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA-) at 37°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol with or without NA (I- NA+ or I- NA-, respectively). The remainder of each culture was grown in allowed to continue growing its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

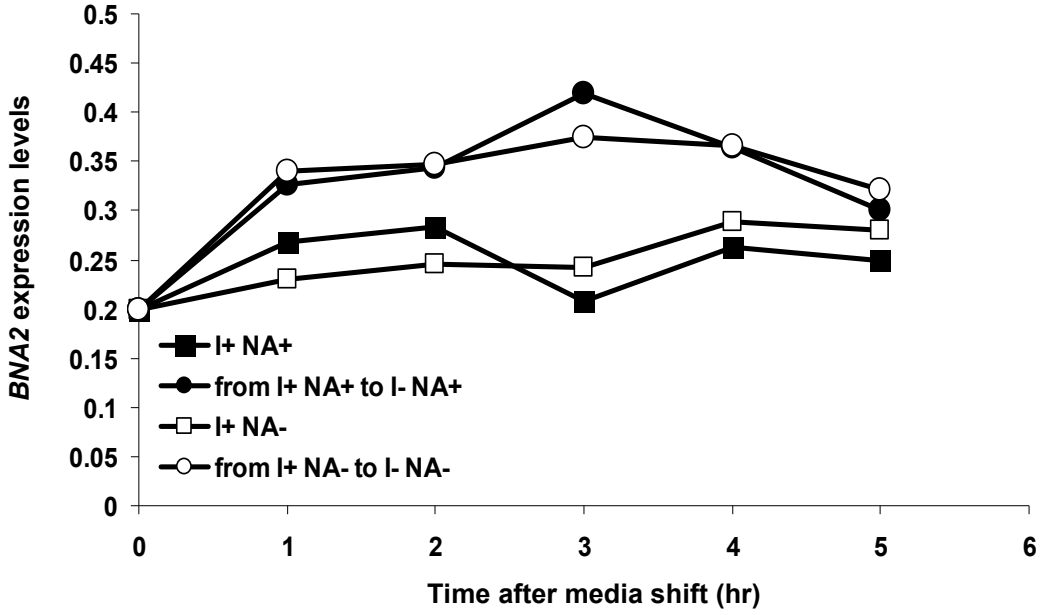


Figure 29. *BNA2* gene expression patterns in the *npt1*Δ strain following a shift to medium lacking inositol at 37°C. Cells were grown in the presence of inositol with or without NA (I+ NA+ or I+ NA-) at 37°C. At mid-logarithmic phase (t=0), half of each culture was shifted to medium lacking inositol with or without NA (I- NA+ or I- NA-, respectively). The remainder of each culture was grown in allowed to continue growing its original medium (I+ NA+ or I+ NA-, respectively). Cells from each culture were harvested at equivalent time intervals. *BNA2* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

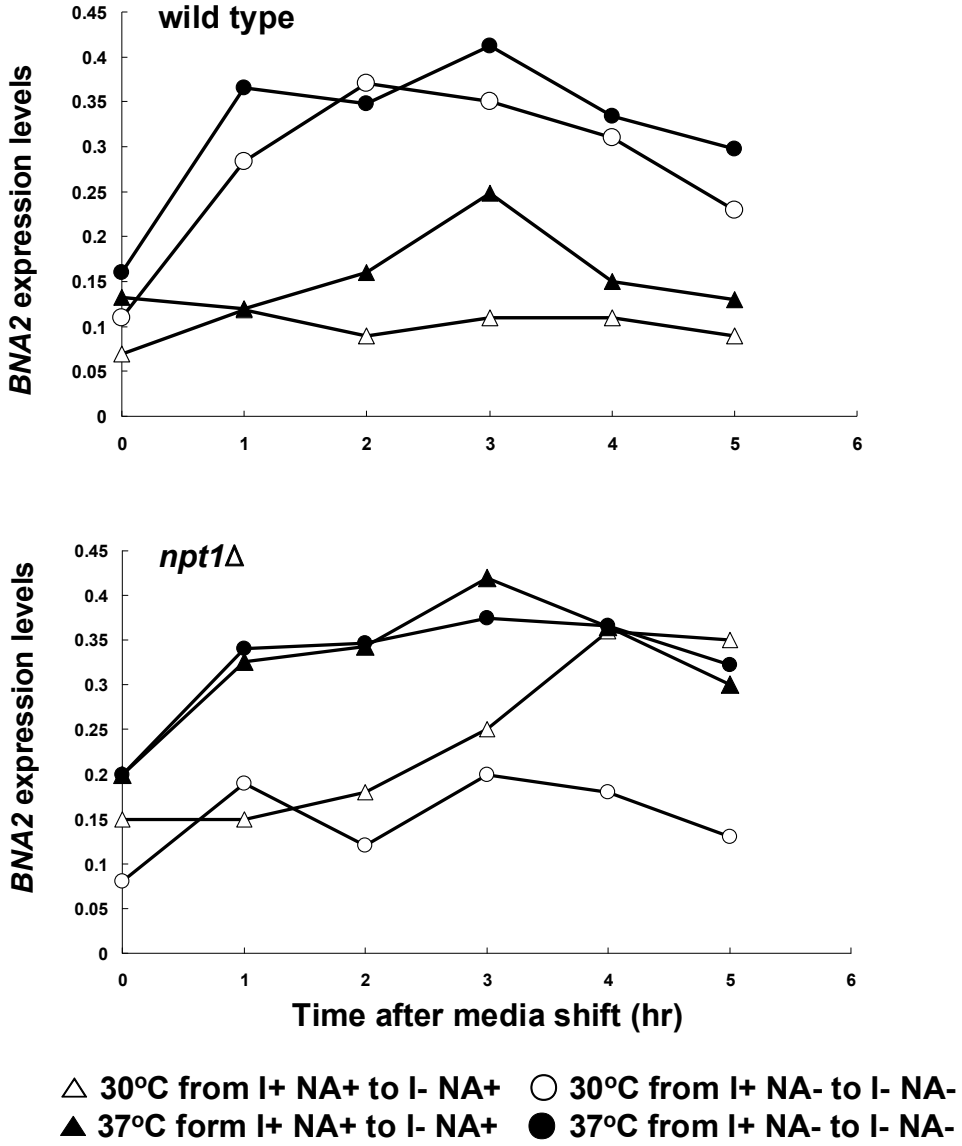


Figure 30. *BNA2* expression levels in *npt1*Δ and wild type cells shifted to inositol free medium at 30 and 37°C. These figures were modified with the results of the pattern of *BNA2* expression as shown in the Figure 23(wild type at 30°C), Figure 25(*npt1*Δ at 30°C), Figure 28(wild type at 37°C) and Figure 29 (*npt1*Δ at 37°C).

B. 12. *TNA1* expression pattern in wild type and *npt1*Δ cells under different growth conditions

In NAD^+ metabolism, there is another regulated gene which not directly involved in either the *de novo* or the salvage pathway, namely the *TNA1* gene, encoding the NA transporter protein, Tna1p, which is plasma membrane permease. It has been reported that the *TNA1* gene in wild type cells is derepressed when NA is absent or at low concentrations in the medium (Klebl, Zillig et al. 2000). Hst1p has been reported to negatively regulate the transcription of *TNA1*, as well as the *BNA2* gene (Bedalov, Hirao et al. 2003).

To examine whether *TNA1* gene expression responds to NA and/or inositol, experiments similar in design to those used to analyze the pattern of *BNA2* expression in wild type and *npt1*Δ cells were employed (Figure 23 and 25). Cells were grown in I+ NA+ or I+ NA- medium at 30°C until mid-logarithmic phase (A_{600}=0.5-0.6). Half of each culture in each condition was shifted to medium lacking inositol with or without NA (I- NA+ or I- NA-). The remainder of each culture was grown continuously in the original medium containing inositol (I+ NA+ or I+ NA-). Cells were harvested at 0, 1 and 3 hrs following the media shift or equivalent interval times in original medium.

As expected, *TNA1* expression levels in wild type cells grown continuously in the absence of NA were significantly higher than in cells grown continuously in the presence of NA, as shown at 0 hr, mid-logarithmic phase (Figure 31). In wild type cells grown in the presence of NA, transcription of *TNA1* gene was repressed. This result was consistent with the previous report by Klebl, *et al*., 2000. However, surprisingly, in cells grown continuously in I+ NA+ medium, *TNA1* gene expression increased gradually over time, whereas *TNA1* expression levels in cells shifted to I- NA+ medium did not much change from the initial level (Figure 31). In contrast, in

the absence of NA, *TNA1* expression levels in cells grown continuously in I+ NA- medium decreased almost 2-fold reaching a level similar to the level seen in the cells grown continuously in I+ NA+ by 3 hrs, whereas transcript levels of *TNA1* in cells transferred to I- NA- medium remained at the high initial levels (Figure 31).

*npt1*Δ cells, under all growth conditions tested, exhibited higher levels of *TNA1* expression compared to the levels in wild type (compare Figure 31 with Figure 32). However, over the course of the experiment, *TNA1* expression levels in *npt1*Δ cells gradually decreased under all growth conditions, as the cells grew out to the 3 hr time point. *TNA1* levels in *npt1*Δ cells grown continuously in I+ NA- medium decreased dramatically, reaching a level 3-fold lower than in cells grown continuously in I+ NA+ medium (Figure 32). However, *TNA1* expression levels in *npt1*Δ cells shifted to I- NA+ or I- NA- medium also gradually decreased.

Summary : *TNA1* expression in wild type cells clearly responds to exogenous NA, increasing *TNA1* expression levels in the absence of NA and decreasing the levels in the presence of NA. However, the *npt1*Δ strain exhibited high *TNA1* expression levels whether inositol and/or NA was present or not. However, the levels gradually decreased over the time.

B. 13. *TNA1* expression levels in the *hst1*Δ strain

As mentioned above, Hst1p reportedly regulates the transcription of both *BNA2* and *TNA1* genes (Bedalov, Hirao et al. 2003). Therefore, *TNA1* expression in *hst1*Δ may reflect the function of Hst1p in gene regulation response to inositol and/or NA. These experiments followed a design similar to that of previous experiments in wild type and *npt1*Δ cells (Figure 31 and 32). Cells grown in I+ NA+ or I+ NA- medium at 30^{o}C until mid-logarithmic phase (A_{600}=0.5-0.6) were transferred to I- NA+ or I- NA- medium, respectively. Cells were harvested at 0, 1, 3, and 6 hrs

following the shift. *TNA1* expression levels in *hst1*Δ cells were measured and compared with the levels in wild type and *npt1*Δ cells under the same conditions.

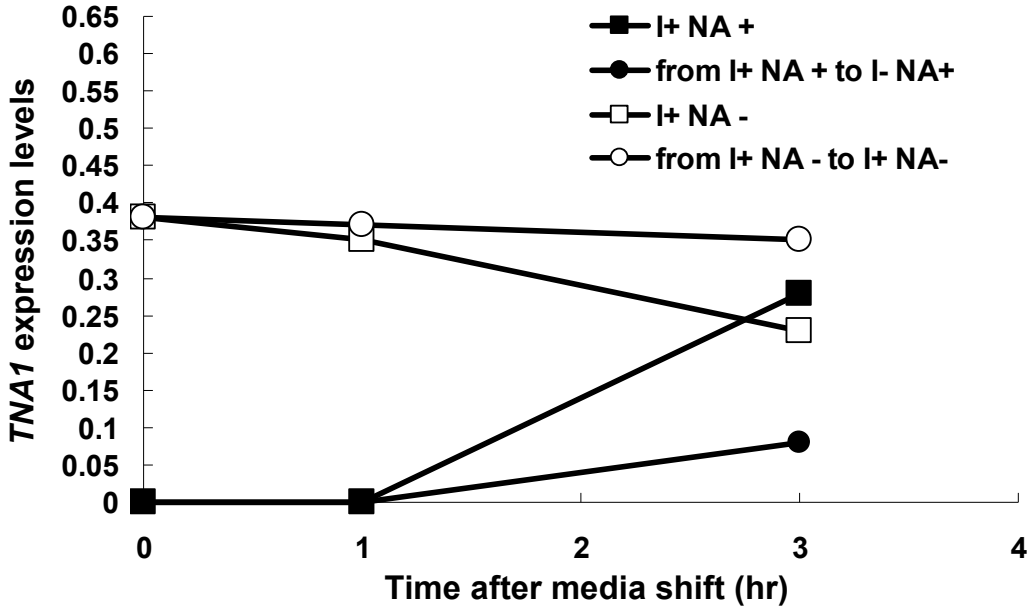


Figure 31. *TNA1* gene expression levels in wild type cells under four different growth conditions. Cells were grown in I+ NA+ or I+ NA- media at 30°C until mid-logarithmic phase (A_{600}=0.5-0.6), t=0. Half of each culture was shifted to I- NA+ (●) or I- NA- (○) medium, respectively. The remainder of each culture was allowed to continue grow in I+ NA+ (■) or I+ NA- (□) medium. Cells were harvested at 0, 1 and 3 hrs following the media shift or an equivalent time in the original culture. *TNA1* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

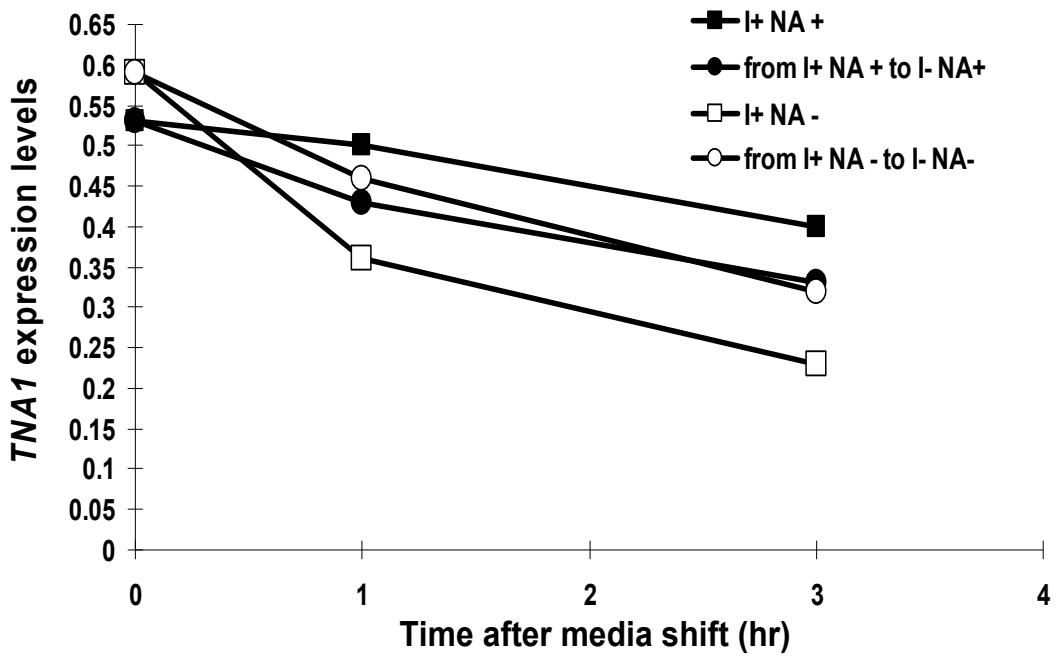


Figure 32. *TNA1* gene expression levels in *npt1*Δ cells under four different growth conditions. Cells were grown in I+ NA+ or I+ NA- medium at 30°C until mid-logarithmic phase (A_{600}=0.5-0.6), t=0. Half of each culture was shifted to I- NA+ (●) or I- NA- (○) medium, respectively. The remainder of each culture was allowed to continue grow in I+ NA+ (■) or I+ NA- (□) medium. Cells were harvested at 0, 1 and 3 hrs following the media shift or an equivalent time in the original culture. *TNA1* mRNA levels were normalized to *ACT1* mRNA levels. The data was obtained from one experiment.

As described previously (Figure 31), wild type exhibited no changes in the initial *TNA1* expression levels following the media shift to I- NA+ or I- NA-; the presence of NA showed very low level, whereas the absence of NA continuously led high *TNA1* expression level (Figure 33). Deletion of *HST1* resulted high *TNA1* expression levels similar to the levels observed in the *npt1*Δ strain at the initial time point (t=0) and at 1hr following the shift to I- NA+ or I- NA- (Figure 33). After 1 hr following the media shift to I- NA+ or I- NA- medium, the *hst1*Δ mutant exhibited consistently high *TNA1*expression levels (Figure 33), in contrast to the pattern of *TNA1* expression in *npt1*Δ cells which gradually decreased over time (Figure 32).

Summary : *TNA1* transcription in the wild type strain responds to exogenous NA. In the absence of NA, high *TNA1* expression levels were observed. However, *TNA1* expression levels in the *hst1*Δ strain were continuously upregulated, whether NA was present or not.

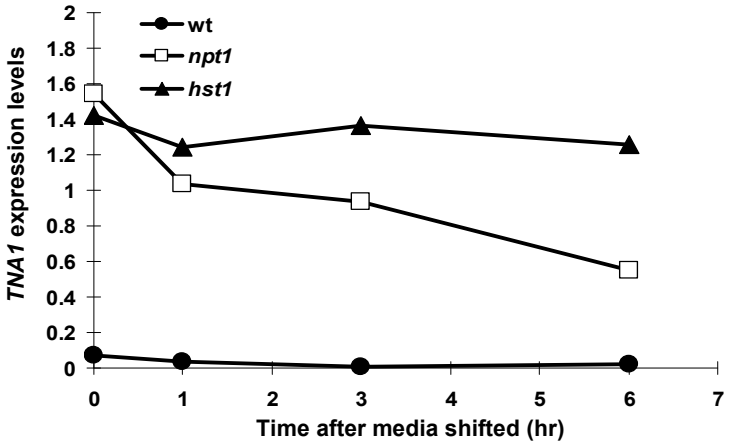
C. The effect of inositol and nicotinic acid (NA) supplementation in phospholipid composition

C. 1. Phospholipid profiles under continuous growth conditions

Exogenous inositol alters the phospholipid composition of yeast cells (Gaspar, Aregullin et al. 2006) and inositol auxotrophy is associated with the alterations in lipid metabolism (Gaspar, Aregullin et al. 2006; Nunez, Jesch et al. 2008). Therefore, it was hypothesized that exogenous NA with and without inositol might result in changes in lipid composition and that the changes in phospholipid metabolism might play a role in the Ino$^-$ phenotype of the *npt1*Δ strain.

The phospholipid and neutral lipid composition of *npt1*Δ and wild type cells

a. Cells were shifted from I+ NA+ to I- NA+ medium



b. Cells were shifted from I+ NA- to I- NA- medium

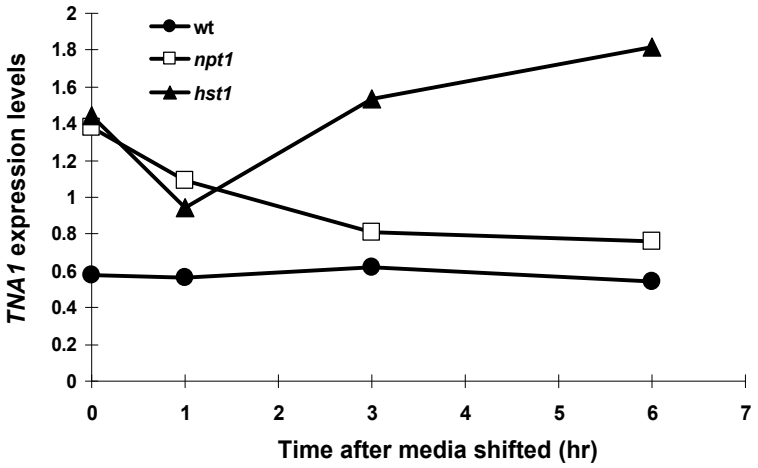
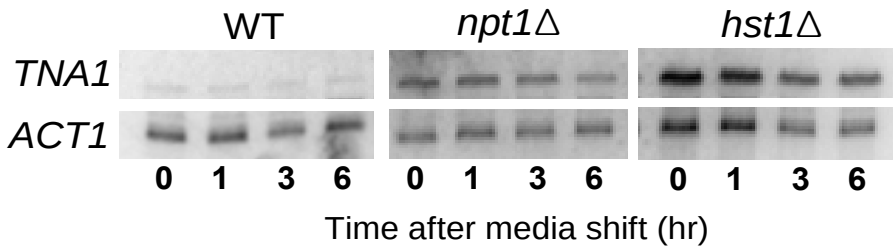


Figure 33. A. *TNA1* gene expression levels in the several strains when cells were shifted to medium lacking inositol. Cells grown in I+ NA+ and I+ NA- medium were shifted to I- NA+ (a) and I- NA- (b) media at mid-logarithmic phase (A_{600}=0.5-0.6), respectively. Wild type (◆), *npt1*Δ (□), and *hst1*Δ (▲) cells were harvested at 0, 1, 3 and 6 hrs following a medium shift. *TNA1* mRNA levels were normalized to *ACT1* mRNA. The data was obtained from one experiment.

a. Cells were shifted from I+ NA+ to I- NA+ medium



b. Cells were shifted from I+ NA- to I- NA- medium

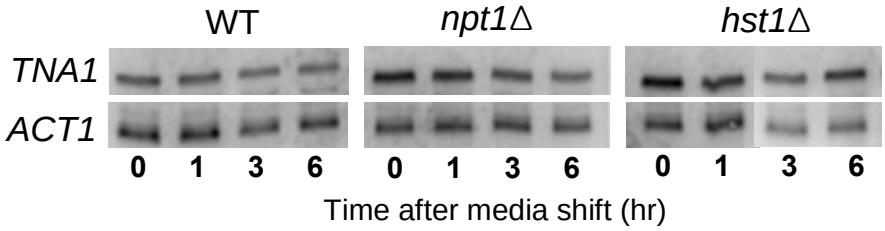


Figure 33. B. Representative Northern blot showing repression of *TNA1* expression in response to NA supplementation requires Hst1p. Inositol has limited or no effect on *TNA1* expression. Cells grown in I+ NA+ and I+ NA- medium were shifted to I- NA+ (a) and I- NA- (b) media at mid-logarithmic phase (A_{600}=0.5-0.6), respectively. Wild type, *npt1*Δ and *hst1*Δ cells were harvested at 0, 1, 3 and 6 hrs following a medium shift. *TNA1* transcript abundance was analyzed by Northern blotting in all strains mention above. *ACT1* transcript level served as a loading control. These blot results were quantified as shown in Figure 33. A.

grown continuously in four different media, I+ NA+, I- NA+, I+ NA- and I- NA- at 30^{o}C were analyzed using steady-state labeling with [1-^{14}C] acetate. This method also allows the total amount of ^{14}C-acetate accumulated in neutral lipids to be quantitatively and compared to the level of the label in phospholipids. Since [1-^{14}C] acetate is incorporated into all carbon-containing metabolites, the proportionate distribution of 1-^{14}C in lipid in cells labeled in this fashion provides an assessment of overall lipid composition (Gaspar, Jesch et al. 2008). Neutral lipid composition will be discussed in a subsequent section.

In wild type cells, as expected, the phospholipid composition changed in response to inositol (I+ versus I-), but exogenous NA (NA+ versus NA-) had little effect (Figure 34.A). The PI content of wild type cells grown in the presence of inositol increased around 3-fold compared with cells grown in the absence of inositol. The PS, PE and total neutral lipid (TNL) levels in wild type cells grown in I- medium increased around 2-fold compared with cells grown in I+ medium. PC content in the absence of inositol also increased markedly, reaching levels approximately 3-fold higher than cells grown in the presence of inositol (Figure 34.A). These results are consistent with published reports, including that of Gaspar *et al* (2006).

In *npt1*Δ cells in the presence of inositol, like wild type cells, there were no significant differences in phospholipid composition whether NA was present or not (Figure 34.B). However, in the absence of inositol, the lipid composition of *npt1*Δ cells showed a significant difference in response to NA. PI level in *npt1*Δ in the presence of inositol was 2-fold higher than the level in the absence of inositol, whether NA was present or not. However, PS levels in *npt1*Δ in I- NA+ medium increased to levels 2.5-fold higher than the levels observed in the presence of inositol. PC levels in *npt1*Δ in the absence of inositol when NA was present increased 3-fold compared to the levels observed in the presence of inositol. Furthermore, when NA was omitted

from the growth medium in the absence of inositol (I- NA- medium), PS and PC levels in *npt1*Δ cells increased 3 or 3.5 fold compared to the levels seen in the presence of inositol , I+ NA+ and I+ NA- media (Figure 34.B).

When the phospholipid composition of the *npt1*Δ strain was compared to that of wild type grown in the presence of NA (I+ NA+ or I- NA+ medium), the levels of all phospholipid constituents in both strains were similar. However, when NA was omitted from the medium in the absence of inositol (I- NA-), PS, PC and TNL levels of *npt1*Δ cells were around 1.5-fold higher than the levels in wild type cells under the same condition (Figure 34.A and B).

Summary : In *npt1*Δ cells, the overall pattern of changes in phospholipid composition in response to inositol was similar to the pattern observed in wild type cells. PI content increased in the presence of inositol, whereas PS, PE, PC and TNL levels increased in the absence of inositol. However, *npt1*Δ cells grown continuously in I- NA- medium exhibited an increase in PC and TNL levels compared to the levels in wild type under the same conditions (Figure 34).

C. 2. Phospholipid analysis of *npt1*Δ and wild type cells shifted from medium containing inositol to medium lacking inositol compared with cells grown continuously in medium lacking inositol

To match the kinetics of changes in the transcription patterns of specific genes involved in the NAD^+ metabolism and inositol biosynthesis with changes in phospholipid composition in each strain, the following analysis was carried out using cells shifted to medium lacking inositol from medium containing inositol. The *INO1*, *BNA2* and *TNA1* gene expression levels were followed assay under these same conditions over an identical time period.

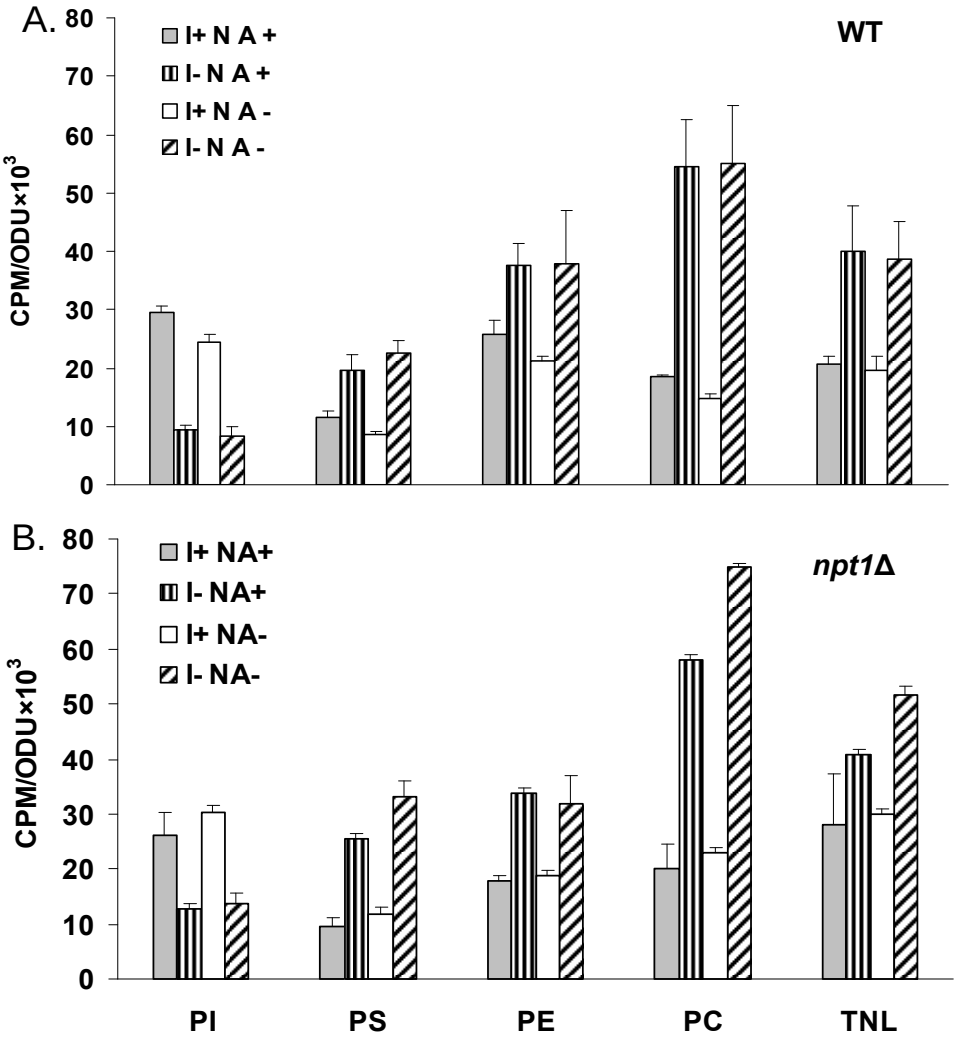


Figure 34. Phospholipid profiles of wild type cells (A) compare with these in *npt1*Δ cells (B) under continuous growth conditions with or without inositol and NA. Cells were grown to steady state in the presence of [1-^{14}C-acetate] in four different media, I+ NA+, I- NA+, I- NA+ and I- NA- at 30^{o}C . Cells were harvested at A_{600}=1.0. Growth conditions, media, labeling and lipid extraction and separation are described in the Materials and Methods. Quantitation was performed by analysis on a Storm 860 PhosphorImager and analyzed with ImageQuant software. Data was average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

Wild type and *npt1*Δ cells grown in I+ NA+ or I+ NA- medium at 30^{o}C until A_{600}=0.5-0.6 were transferred to medium lacking inositol with or without NA, respectively (I- NA+ or I- NA- medium). Cells were then harvested at 3 hrs following the media shift, around A_{600}=1.0. The phospholipid analysis was conducted using steady-state [1-^{14}C]-acetate labeling as described in the Materials and Methods. The phospholipid composition of the cells was compared with the cells grown continuously in medium lacking inositol (I- NA+ or I- NA- medium).

When wild type cells were shifted for 3 hrs from I+ NA+ to I- NA+ or from I+ NA- to I- NA- medium, PI and TNL levels reached levels observed in cells grown continuously in I- NA+ or I- NA- medium. However, PS, PE and PC levels in wild type cells shifted to medium lacking inositol decreased around 40 or 50% compared to the levels in cells grown continuously in I- NA+ or I- NA- medium, whether NA was present or not (Figure 35), but the levels were still higher than the levels grown continuously in medium containing inositol (Figure 34.A and 35).

In contrast to wild type cells, *npt1*Δ cells shifted to medium lacking inositol exhibited a difference in response to the presence or absence of NA. When *npt1*Δ cells were shifted from I+ NA+ to I- NA+ medium, PE and PC levels decreased just 20-30% compared to the levels observed in cells grown continuously in I- NA+ medium. However, when *npt1*Δ cells were shifted from I+ NA- to I- NA- medium, PS, PE and PC levels decreased 40-50% compared to the levels in cells grown continuously in I- NA- medium like wild type (Figure 36). Therefore, PC levels in *npt1*Δ cells shifted from I+ NA+ to I- NA+ were higher than the levels in wild type cells under the same condition because PC level in *npt1*Δ cells decreased less than in wild type (Figure 35 and 36). However, when *npt1*Δ cells were shifted from I+ NA- to I- NA- medium, the phospholipid composition was similar to wild type cells grown under the same condition (Figure 35 and 36).

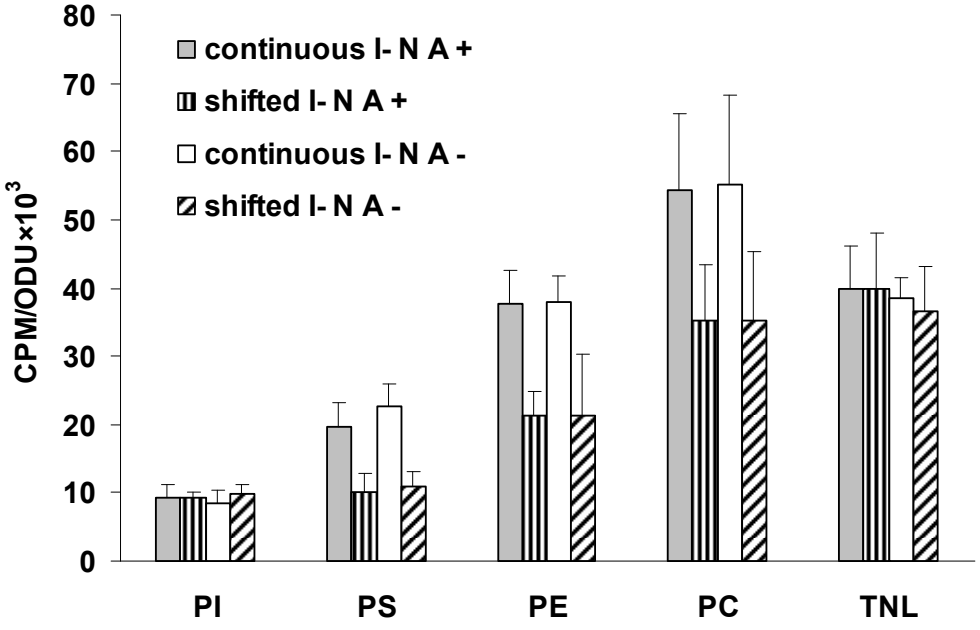


Figure 35. Phospholipid profiles of wild type cells shifted to medium lacking inositol compared with the cells grown continuously in medium lacking inositol. Cells were grown in presence of inositol (I+ NA+ and I+ NA-) and then were shifted to the medium lacking inositol (I- NA+ and I- NA-) at mid-logarithmic phase (A_{600}=0.5-0.6). Cells were harvested at 3 hrs following the media shift, around A_{600}=1.0. Alternatively, cells were continuously grown in I- NA+ and I- NA- until A_{600}=1.0. Extraction of lipids and separation of phospholipid species is described in the Materials and Methods section. Quantitation was performed by analysis on a Storm 860 PhosphorImager and analyzed with ImageQuant software. Data was average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

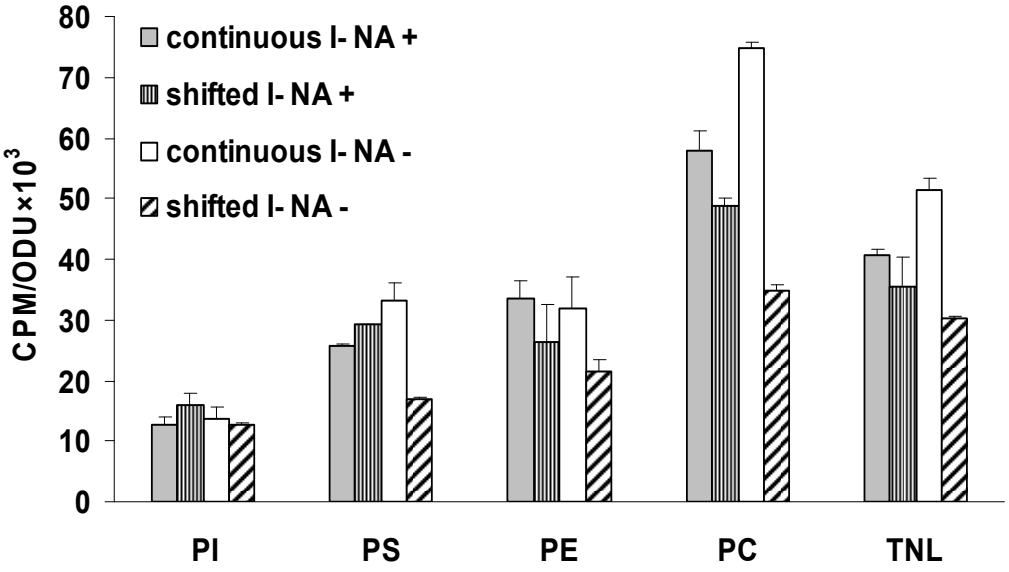


Figure 36. Phospholipid profiles in the *npt1*Δ cells shifted to medium lacking inositol compared with the cells grown continuously in medium lacking inositol. Cells were grown in presence of inositol (I+ NA+ and I+ NA-) and then were shifted to the medium lacking inositol (I- NA+ and I- NA-) at mid-logarithmic phase, A_{600}=0.5-0.6. Cells were harvested at 3 hrs following the media shift, around A_{600}=1.0. Alternatively, cells were continuously grown in I- NA+ and I- NA- until A_{600}=1.0. Extraction of lipids and separation of phospholipid species is described in the Materials and Methods section. Quantitation was performed by analysis on a Storm 860 PhosphorImager and analyzed with ImageQuant software. Data was average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

Summary : Increased PC levels were observed in *npt1*Δ cells shifted to I- NA+ medium compared to wild type, but the PC levels in wild type and *npt1*Δ were similar when the cells were shifted from I+ NA- to I- NA- medium.

C. 3. Neutral lipid analysis in the *npt1*Δ and wild type strains under continuous growth conditions

The neutral lipids analyzed include 1,2-diacylgylcerol (1,2-DAG), free sterol, free fatty acid (FFA), triacylglycerol (TAG) and sterol ester (SE). Cells were grown in four different media, I+ NA+, I- NA+, I+ NA- and I- NA- at 30^{o}C in the presence of ^{14}C-acetate to steady state as described in the Materials and Methods until they reached A_{600}=1.0. Neutral lipid composition was analyzed in cells grown under the same conditions as used for analysis of phospholipids. After lipids were extracted, neutral lipids were analyzed as described in the Materials and Methods.

In the analysis of phospholipid compositions reported in the previous section, total neutral lipid (TNL) levels in wild type cells were observed to increase 2-fold in the absence of inositol (I- NA+ and I- NA-) compared to the levels observed in the presence of inositol (I+ NA+ and I+ NA-) (Figure 34. A). TNL levels in *npt1*Δ cells grown continuously in I- NA+ medium increased in a fashion similar to the increase seen in wild type under the same condition. However, in *npt1*Δ, the increase in TNL levels in I- NA- medium was greater than the levels in I- NA+ medium (Figure 34. B).

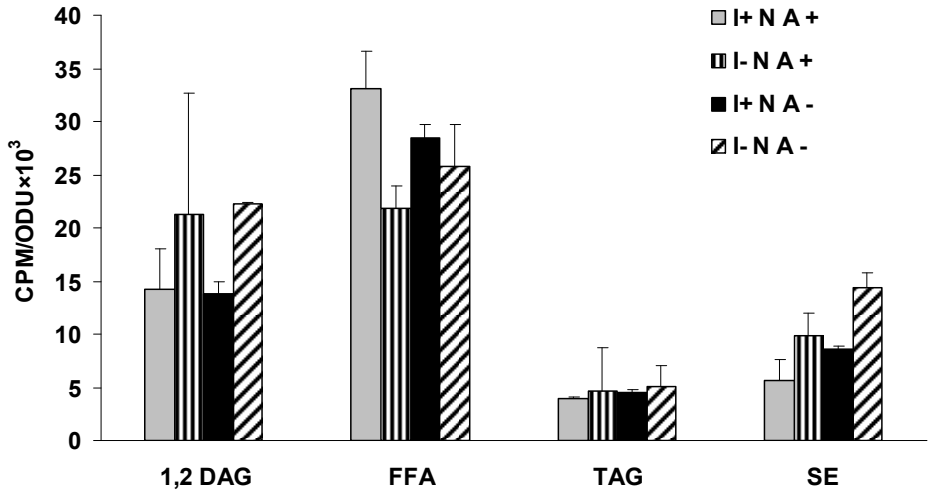
Upon separation of the individual neutral lipids, 1,2-diacylgylcerol (1,2-DAG) levels in wild type cells grown in the absence of inositol (I- NA+ and I- NA-) were seen to increase around 1.5-fold in comparison to the levels observed in cells grown in the presence of inositol (I+ NA+ and I+ NA-) whether NA was present or not. Free fatty acid (FFA) levels in wild type cells grown in I+ NA+ medium were higher than in cells grown in I- NA+ medium as previously reported by Gaspar *et al* (2006).

However, in wild type cells in the absence of NA, there was no significant change in FFA levels in response to inositol (I+ NA- and I- NA- media). Triacylglycerol (TAG) levels in wild type were unchanged under all four different growth conditions. Sterol ester (SE) levels in wild type cells grown in the absence of inositol (I- NA+ and I- NA- media) were higher than the levels observed in cells grown in the presence of inositol (I+ NA+ and I+ NA- media) (Figure 37. A).

In *npt1*Δ cells, an increase in 1,2-DAG levels in I- NA+ medium was observed compared to the levels in I+NA+ medium. However, in the absence of NA, no change in 1,2-DAG levels in *npt1*Δ cells was seen in response to inositol. FFA levels in *npt1*Δ in the presence of inositol were significantly higher than in the absence of inositol whether NA was present or not. TAG and SE levels in *npt1*Δ cells were not significantly different in any growth media tested, I+ NA+, I- NA+, I+ NA- and I- NA- (Figure 37. B).

In the presence of inositol, 1,2-DAG levels in wild type were similar to the levels in *npt1*Δ cells. However, in the absence of inositol, 1,2-DAG levels in wild type were 1.4-fold higher than those seen in *npt1*Δ cells. 1,2-DAG levels in wild type responded to inositol in both presence and absence of NA, whereas 1,2-DAG levels in *npt1*Δ cells responded to inositol only in the presence of NA. FFA levels in wild type cells under all of growth conditions tested except I+ NA- increased around 1.3-fold compared to the level in *npt1*Δ cells under the same conditions. Both wild type and *npt1*Δ cells exhibited relatively low TAG levels under four different growth media. Although *npt1*Δ cells exhibited low SE levels, which were similar in all growth conditions tested, SE levels in wild type cells increased in I- medium compared to the level in I+ medium. Notably, SE levels in wild type cells grown in I- NA- medium were 3-fold higher than the levels in *npt1*Δ in I- NA- medium.

A. wild type cells



B. *npt1*Δ cells

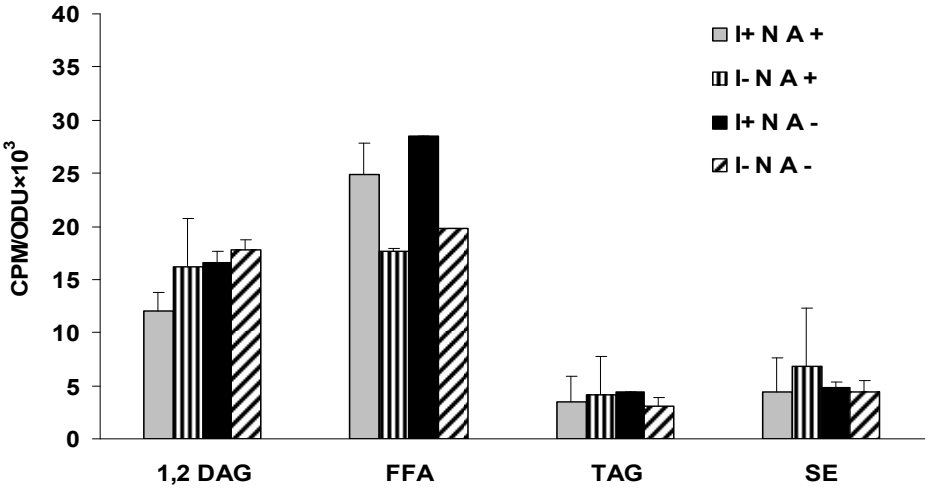


Figure 37. Neutral lipid profiles in the wild type strain (A) compared with *npt1*Δ cells (B) under continuous growth conditions at 30°C. Cells were grown in four different growth media, I+ NA+, I- NA+, I- NA+ and I- NA- until A_{600}=1.0 in which cells were harvested as described in the Materials and Methods. 1,2 DAG, 1,2 diacyl glycerol; FFA, Free fatty acid; TAG, triacylglycerol; SE, sterol ester. Data represents the average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

Summary : The wild type strain exhibited significant differences in 1,2-DAG and SE levels, depending on the presence or absence of inostiol whether NA was present or not. However, the *npt1*Δ strain did not much differ in 1,2-DAG and SE levels between the presence and absence of inositol. FFA levels in *npt1*Δ significantly increased in the presence of inositol compared to the absence of inositol.

C. 4. Neutral lipid analysis of the wild type and *npt1*Δ strains shifted to medium lacking inositol compared with the cells grown continuously in medium lacking inositol

Cells were grown in I+ NA+ or I+ NA- medium at 30^{o}C until A_{600}=0.5-0.6 and then shifted to medium lacking inositol, I- NA+ or I- NA-. Cells were harvested at 3 hrs following the media shift, around A_{600}=1.0. The neutral lipids (NL) of cells shifted to medium lacking inositol were compared to those observed in cells grown continuously in medium lacking inositol (I- NA+ or I- NA-). The experimental design used for growth and harvesting of cells for neutral lipid analysis were consistent with the methods used for phospholipid lipid analysis.

When wild type cells were shifted to I- NA+ or I- NA- medium, no difference in TNL levels in phospholipid analysis was observed in cells grown continuously in I- NA+ or I- NA- medium, whether NA was present or not (Figure 35). However, wild type cells shifted to medium lacking inositol for 3 hrs exhibited significant differences in several neutral lipid compositions compared to cells grown continuously in medium lacking inositol. Surprisingly, FFA levels in cells shifted to I- NA+ or I- NA- medium were 80% lower than in cells grown continuously in I- NA+ or I- NA- medium (Figure 38). These results were also consistent with the results by Gaspar *et al*., 2009, unpublished data. SE levels in wild type cells shifted to I- NA+ or I- NA- for 3 hrs

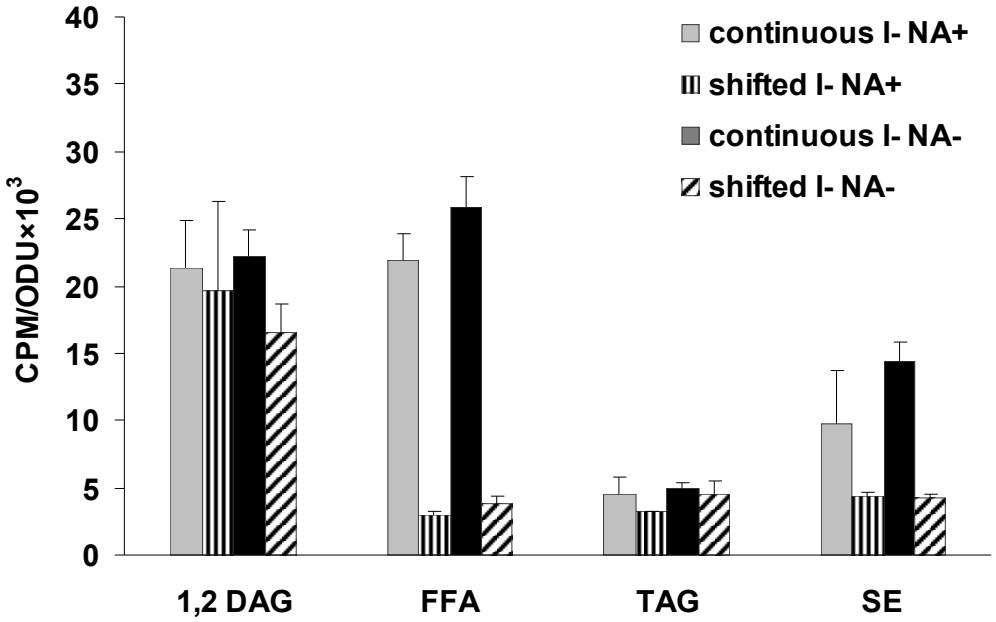


Figure 38. Neutral lipid analysis in wild type following a shift to medium lacking inositol compared with the cells grown continuously in I- NA+ or I- NA- medium. Cells grown in I+ NA+ or I+ NA- medium were shifted to absence of inositol (I- NA+ and I- NA-) at mid-logarithmic phase (A_{600}=0.5-0.6). Cells were then harvested at 3 hrs following media shift, around A_{600}=1.0. Alternatively, cells were continuously grown in I- NA+ or I- NA- medium until A_{600}=1.0. Extraction of lipids and separation of neutral lipid species are described in the Materials and Methods. 1,2-DAG, 1,2 diacyl glycerol; FFA, Free fatty acid; TAG, triacylglycerol; SE, sterol ester. Data was average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

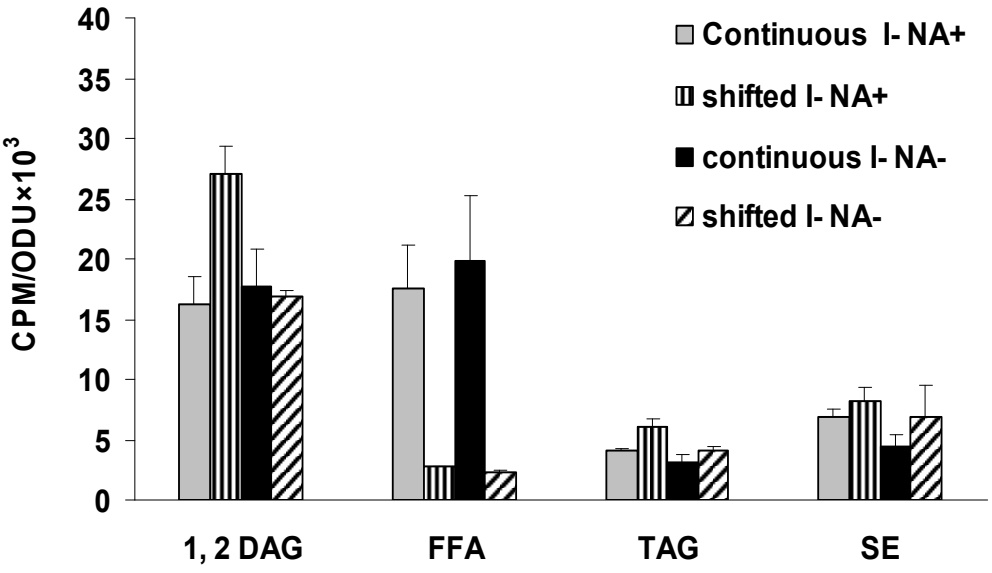


Figure 39. Neutral lipid analysis in the *npt1*Δ strain following a shift to medium lacking inositol compared with the cells grown continuously in I- NA+ or I- NA- medium. Cells grown in I+ NA+ or I+ NA- medium were shifted to medium lacking inositol (I- NA+ or I- NA-, respectively) at mid-logarithmic phase (A_{600}=0.5-0.6). Cells were then harvested at 3 hrs following media shift, around A_{600}=1.0. Alternatively, cells were grown continuously in I- NA+ or I- NA- medium until A_{600}=1.0. Extraction of lipids and separation of neutral lipid species are described in the Materials and Methods. 1,2-DAG, 1,2 diacyl glycerol; FFA, Free fatty acid; TAG, triacylglycerol; SE, sterol ester. Data was average from three independent experiments and the error bars represent the standard deviation (S.D), n=3.

were 2-3 fold lower than the levels in cells grown continuously in I- NA+ or I- NA- medium (Figure 38). However, no changes in 1,2-DAG and TAG levels were observed under any of the growth conditions mentioned above (Figure 38).

When *npt1*Δ cells were transferred to medium lacking inositol, changes in neutral lipid compositions in response to NA supplementation were observed. 1,2-DAG levels in *npt1*Δ cells shifted from I+ NA+ to I- NA+ medium increased about 2-fold compared to the level observed in *npt1*Δ cells shifted from I+ NA- to I- NA- medium and grown continuously in I- NA+ and I- NA- media. FFA levels were dramatically decreased when cells were shifted to I- medium. Like wild type cells, FFA levels in *npt1*Δ cells shifted to I- NA+ or I- NA- medium were about 4-fold lower than in cells grown continuously in I- NA+ or I- NA-. TAG levels in *npt1*Δ cells shifted to I- NA+ medium were higher than the levels under other conditions. However, no significant difference in SE levels was detected in cells shifted to I- medium compared to levels in cells grown continuously in I- medium (Figure 39).

Summary : Interestingly, 1,2-DAG and TAG levels in *npt1*Δ cells shifted to medium lacking inositol with NA (I- NA+) increased compared to the levels in *npt1*Δ cells under other conditions tested, as well as, in comparison to the levels observed in wild type cells under the same conditions. The 1,2-DAG and TAG levels in *npt1*Δ cells were similar to levels in wild type when cells were shifted to I- NA- medium.

CHAPTER FOUR
DISCUSSION & CONCLUSION

A. The Ino$^-$ phenotype of *npt1*Δ is suppressed or enhanced by several effectors

A. 1. The effects of supplementation with various metabolites on the Ino$^-$ phenotype of the *npt1*Δ strain

As described in the Introduction, the *npt1*Δ mutation eliminates NAD^+ synthesis by blocking the salvage pathway (Bedalov, Hirao et al. 2003) (Figure 2.A). In these results, it was observed that the *npt1*Δ mutant exhibits a weak Ino$^-$ phenotype at 37^{o}C (Figure 3.A and Table 5).

The strength of the Ino$^-$ phenotype of *npt1*Δ containing strains was affected by the presence in the growth medium of several metabolites involved in NAD^+ metabolism. The presence of both NA and NAM caused the phenotype to strengthen in *npt1*Δ, *npt1*Δ*hst1*Δ and *npt1*Δ*pnc1*Δ strains, whereas the absence of tryptophan resulted in slightly increased growth in the absence of inositol. The Ino$^-$ phenotype was partially suppressed by removal of NA from the media at 37^{o}C (Figure 4). This result is somewhat surprising since NA cannot be used as a precursor of NAD^+ in *npt1*Δ cells. Therefore, it was speculated that exogenous NA might result in increased cellular levels of NA, which in turn might affect NAD^+ metabolism. However, intracellular NA levels in *npt1*Δ were not significantly affected by exogenous NA (Figure 21). This topic will be discussed further in a subsequent section of the Discussion. Addition of 5 mM NAM, an inhibitor of sirtuins, such as Sir2p, Hst1p and Hst2p, which are NAD^+-dependent protein deacetylases (Bitterman, Anderson et al. 2002), also resulted in a stronger Ino$^-$ phenotype at 37^{o}C in all strains carrying the *npt1*Δ mutation, whether NA was present or not. Furthermore, the presence of NAM

also resulted in a weak Ino$^-$ phenotype in the *npt1*Δ strain even at 30^{o}C (Figure 5). Tryptophan is a precursor of NAD$^+$ synthesis via the *de novo* pathway (Figure 2.A) and the synthetic complete (SC) medium routinely used contains tryptophan (See Materials and Methods). Omitting tryptophan from growth media had a relatively small suppressive effect on the Ino$^-$ phenotype of double mutants carrying the *npt1*Δ mutation (i.e., *pnc1*Δ *npt1*Δ and *hst1*Δ *npt1*Δ) (Figure 7), but did not appear to affect the Ino$^-$ phenotype of the *npt1*Δ single mutant. However, it is important to point out that all strains used in this study are capable of synthesizing tryptophan. Therefore, the absence of exogenous tryptophan by itself should not affect viability of wild type cells. However, it is possible that cells relying on endogenous synthesis of tryptophan would have lower levels of intracellular tryptophan and that lower intracellular levels of tryptophan could reduce the cellular capacity to synthesize NAD$^+$ *de novo*. Consistent with this concept, it is reported that NAD$^+$ levels in wild type cells are reduced in the absence of exogenous tryptophan (Bedalov, Hirao et al. 2003).

Provision of exogenous choline, a major phospholipid precursor, also resulted in a stronger Ino$^-$ phenotype in all of the mutant strains tested in this study that show a partial Ino$^-$ phenotype at 37^{o}C (Figure 6). The presence of choline has been shown to strengthen the Ino$^-$ phenotype of certain mutants that are defective in specific cellular stress responses (Nunez, Jesch et al. 2008). The mechanism of the choline effect on the Ino$^-$ phenotype of *mpk1*Δ, defective in the Cell Wall Integrity Protein Kinase C (CWI-PKC) signaling and other CWI-PKC pathway mutants are discussed in Nunez *et al*., 2008. It is thought to involve competition for key intermediates in phospholipid and neutral lipid metabolism, such as PA and DAG. This topic will be discussed in the next section of the Discussion dealing with lipid metabolism in the *npt1*Δ mutant.

Removal of threonine from the medium also strengthened the relatively weak Ino$^-$ phenotype of the *npt1*Δ and *pnc1*Δ *npt1*Δ strains at 37^{o}C (Figure 8). A similar

effect of threonine on the Ino$^-$ phenotype of several mutants with defects in stress response pathways has been observed in our lab (M. Villa-Garcia and S. Henry, unpublished data), but the mechanism of this effect is not known.

Conclusion : The Ino$^-$ phenotype of the *npt1*Δ strain is affected by addition of metabolites involved in the NAD^+ metabolism, especially NA and NAM, suggesting a role for sirtuins activity in the Ino$^-$ phenotype. The effect of choline on the Ino$^-$ phenotype of the *npt1*Δ strain suggests the possibility that changes in phospholipid composition and/or cellular stress responses may contribute to the phenotype.

A. 2. The Ino$^-$ phenotype of the *npt1*Δ strain is enhanced by the *hst1*Δ mutation

Deletion of the *HST1* gene in the *npt1*Δ strain resulted in a stronger Ino$^-$ phenotype at 37^{o}C (Figure 3. B). However, the *hst1*Δ mutation by itself did not result in a growth defect under any of the conditions tested (Figure 3.A). Hst1p has been specifically implicated as a sensor of NAD^+ levels and a regulator through the repression of the *BNA* genes in the *de novo* NAD^+ pathway (Bedalov, Hirao et al. 2003). As mentioned above, addition of NAM resulted in a considerable strengthening of the Ino$^-$ phenotype of the *npt1*Δ strain, such that it was evident at 30^{o}C as well as 37^{o}C (Figure 5). Taken together, these findings suggest that the Ino$^-$ phenotype of the *npt1*Δ mutant may be influenced by changes in the activity of sirtuins.

Cells lacking the sirtuin, Hst1p, show constitutively high expression of the *de novo* NAD^+ pathway genes including *BNA2*, suggesting that Hst1p may play an important role in signaling low NAD^+ levels. However, the presence of exogenous NAM provided an even stronger effect on the weak Ino$^-$ phenotype of the *npt1*Δ mutant, as compared to the effect of the deletion of *HST1*. Perhaps this is due to the fact that NAM also inhibits other sirtuins, such as Sir2p and Hst2p, as well as Hst1p (Figure 5). It has been reported that *npt1*Δ strains exhibits low Sir2p activity resulting

in loss of telomeric and rDNA silencing and that *SIR2* overexpression restores rDNA silencing in *npt1*Δ (Sandmeier, Celic et al. 2002). Silencing is a considered to be "proxy" for overall sirtuin activity in the cell (i.e., when NAD^+ levels are low or where sirtuins are inhibited; silencing is lost). However, the *hst1*Δ and *sir2*Δ mutants did not exhibit an Ino^- phenotype (Figure 3.A). Furthermore, the *sir2*Δ mutation had no effect on the phenotype of *npt1*Δ, and only the *hst1*Δ mutation affected the phenotype of *npt1*Δ. Thus, loss of the activity of each of these sirtuins individually does not result in an Ino^- phenotype (Figure 3.A).

Conclusion : The activity of sirtuins appears to be somehow involved in the Ino^- phenotype of the *npt1*Δ strain as shown by strengthening of the phenotype by deletion of *HST1* or addition of exogenous NAM.

A. 3. *NTE1* high copy suppression of the Ino^- phenotype of *npt1*Δ

When *HST1* or *SIR2* highcopy plasmids were introduced into *npt1*Δ cells, no changes in the *npt1*Δ Ino^- phenotype were observed (Figure 9). Thus, while deleting *HST1* did strengthen the *npt1*Δ Ino^- phenotype somewhat, its overexpression was not able to counteract the phenotype. The *ACC1-794* and *SNF4-204* dominant mutations cuase a lower activity of Acc1p (acetyl CoA carboxylase) and are known to suppress the Ino^- phenotypes of a number of mutants that have reduced *INO1* expression. The suppression mechanism is known to involve increased *INO1* expression, although the mechanism by which reduced Acc1p activity influences *INO1* transcription is not entirely understood (Shirra and Arndt 1999; Shirra, Patton-Vogt et al. 2001). However, when low copy (*CEN*) plasmids carrying the dominant mutations, *ACC1-794* and *SNF4-204*, were introduced into *npt1*Δ cells, there was little change in the Ino^- phenotype (Figure 10.A), indicating that any change in *INO1* expression caused by these suppressors was insufficient to suppress the *npt1*Δ phenotype.

However, transformation with a highcopy plasmid carrying the *NTE1* gene did suppress the Ino$^-$ phenotype of *npt1*Δ (Figure 10.B). Nte1p, an endoplasmic reticulum (ER)-localized phospholipase B, is known to mediate phosphatidylcholine (PC) turnover (Figure 1.C) in the presence of exogenous choline as well as at high growth temperature (37^{o}C) (Zaccheo, Dinsdale et al. 2004). *NTE1* highcopy expression has been shown to suppress the inositol auxotrophy of the *mpk1*Δ mutant, which is defective in CWI-PKC signaling (Nunez, Jesch et al. 2008) (Fernandez-Murray J.P., *et al*, 2009).

The Ino$^-$ phenotype of the *mpk1*Δ mutant, like that of *npt1*Δ, is strengthened by higher growth temperatures and the presence of choline (Figure 6). The choline sensitivity of the Ino$^-$ phenotype of *npt1*Δ cells is accompanied by an increase in PC levels compared to wild type, when cells were shifted from I+ NA+ to I- NA+ media (Figure 37). The fact that the Ino$^-$ phenotype of *npt1*Δ was partially rescued by the *NTE1* highcopy plasmid at 37^{o}C (Figure 10.B) suggests that increasing PC levels in the ER, or other cellular membranes, plays a role in the *npt1*Δ Ino$^-$ phenotype. In this regard, the phenotype of the *npt1*Δ mutant resembles that of the *mpk1*Δ mutant (Nunez, Jesch et al. 2008), in which elevated PC levels and defects in PC turnover results in a disruption of PC homeostasis in cellular membranes. CWI-PKC signaling is a key cellular stress response needed for survival at high temperatures, low osmolarity and in the absence of inositol. Mutants in several other stress responses, such as the *hac1*Δ and *ire1*Δ mutants, defective in the Unfolded Protein Response (UPR) signaling pathway (Chang, Jones et al. 2002) and the high osmolarity glycerol (HOG) pathway (Manuel Villa., *et al*., unpublished data) also have similar phenotypes. Therefore, the phenotype observed in the *npt1*Δ mutant could indicate that cellular stress responses are affected by deficiency in cellular NAD^+ metabolism.

Conclusion : The Ino$^-$ phenotype of *npt1*Δ is strengthened at higher growth

temperatures and/or the addition of NA, NAM and choline to the growth medium, as well as, by the deletion of *hst1*Δ. The phenotype is partially suppressed by overexpression of *NTE1*, encoding a phospholipase B involved in PC turnover in the ER. The effect of *NTE1* overexpression together with the observation that the presence of exogenous choline strengthens the Ino$^-$ phenotype of *npt1*Δ suggests that build up of PC in the ER may contribute to the phenotype.

B. The weak Ino$^-$ phenotype of *npt1*Δ is associated with a decrease in *INO1* expression and changes in phospholipid and neutral lipid metabolites

B. 1. *INO1* expression levels related to the Ino$^-$ phenotype of the *npt1*Δ strain

Inositol, an essential precursor of eukaryotic phospholipids, is synthesized *de novo* from glucose-6-phosphate in a reaction catalyzed by Ino1p, encoded by the *INO1* gene (Carman and Henry 1999). Based on the Ino$^-$ phenotype described above, I hypothesized that alteration in NAD^+ metabolism might affect expression of *INO1*, the structural gene for inositol-3-phosphate synthase. Alternatively, NAD^+ levels might directly influence the activity of Ino1p. The *INO1* gene was first identified in yeast (Culbertson, Donahue et al. 1976; Donahue and Henry 1981), but homologues exist virtually all eukaryotes studied. In yeast, expression of the *INO1* gene is highly dependent on growth stage and inositol supplementation (Hirsch and Henry 1986; Lamping, Luckl et al. 1994). *INO1* transcription increases in the absence of inositol in response to changing phospholipid composition (Henry and Patton-Vogt 1998; Loewen, Gaspar et al. 2004). As described in the Introduction, the absence of inositol results in an increased level of phosphatidic acid (PA), which binds Opi1p, a negative regulator of *INO1* gene (Loewen, Gaspar et al. 2004) (Figure 1.B). Addition of inositol to the growth medium results in a rapid increase in phosphatidylinositol (PI) synthesis

resulting in depletion of its precursor, phosphatidic acid (PA) (Figure 1.C). Decreased PA levels lead to translocation of the Opi1p repressor from the ER to the nucleus, causing repression of *INO1* (Loewen, Gaspar et al. 2004) (Figur 1.B). The Ino$^-$ phenotype is observed in mutants unable to derepress *INO1*, as well as in strains carrying mutations which inactivate Ino1p (Culbertson and Henry 1975; Donahue and Henry 1981).

Therefore, it was questioned whether the Ino$^-$ phenotype of the *npt1*Δ mutant was caused by the failure of *INO1* expression and whether NA availability in the growth medium and/or increasing temperature affects *INO1* expression. It was found that *INO1* expression levels in the *npt1*Δ and wild type strains were unaffected by the presence or absence of NA at 30^{o}C when cells were shifted from I+ to I- medium holding NA concentration constant (Figure 11 and 13). However, at 37^{o}C, the *INO1* expression level in wild type was 30% lower in cells shifted from I+ NA- to I- NA- medium in comparison to cells shifted from I+ NA+ to I- NA+ medium (Figure 14). In the absence of NA, *INO1* expression levels in *npt1*Δ were comparable to wild type cells shifted to I- NA- medium at 37^{o}C (Figure 14). However, in the presence of NA at 37^{o}C, *npt1*Δ cells exhibited 50% lower *INO1* expression levels than wild type cells at 3hrs following the media shifted from I+ NA+ to I- NA+ medium (Figure 14).

Therefore, a weak Ino$^-$ phenotype of the *npt1*Δ strain at 37^{o}C in the presence of NA is associated with reduced *INO1* expression levels in comparison to wild type. Thus, lower *INO1* expression levels may explain, at least in part, the Ino$^-$ phenotype of the *npt1*Δ strain at 37^{o}C. However, the presence of NA clearly did not lead to full repression of *INO1* expression in the *npt1*Δ mutant when it was shifted to I- NA+ medium at 37^{o}C. Furthermore, although the expression level of *INO1* in the absence of NA in I- medium at 37^{o}C in the *npt1*Δ strain was equivalent to the wild type level at this temperature (Figure 14), the Ino$^-$ phenotype of *npt1*Δ was not fully suppressed

under these conditions, i.e., in the absence of NA (Figure 4). Clearly, *npt1*Δ cells do not grow as well as wild type cells under these conditions.

Like the *npt1*Δ strain, not all mutants that show the Ino$^-$ phenotypes have defects in *INO1* expression levels and phospholipid composition. An example is the *mpk1*Δ mutant defective in the CWI-PKC signaling pathway which has an Ino$^-$ phenotype in the presence of exogenous choline and/or at 37^{o}C. However, *mpk1*Δ has no defect in its ability to derepress *INO1* levels at high temperatures or in the presence of choline (Nunez, Jesch et al. 2008). The *hac1*Δ and *ire1*Δ mutants, defective in the UPR signaling pathway, also have an Ino$^-$ phenotype despite their ability to derepress *INO1*, at least initially, following transfer to I- medium (Cox, Chapman et al. 1997; Chang, Jones et al. 2002). The *sac1*Δ mutant, defective in phosphoinositide phosphatase activity, was also reported to express *INO1* to wild type levels despite its Ino$^-$ phenotype (Rivas, Kearns et al. 1999). These mutants all exhibit defects in stress responses and/or signaling, necessary for survival in medium lacking inositol, especially at higher temperatures.

Conclusion : The Ino$^-$ phenotype of *npt1*Δ is not fully explained by a failure of *INO1* expression.

B. 2. Phospholipid and neutral lipid metabolism is altered in the *npt1*Δ strain in response to inositol and NA

Inositol auxotrophy is also associated with alterations in phospholipid metabolism. The presence of phospholipid precursors, inositol and/or choline in the growth medium, influences phospholipid metabolism in yeast even in wild type cells. In wild type cells, phosphatidylinositol (PI) levels in the presence of inositol were shown to be elevated 3 or 4-times compared to the levels in cells grown in the absence of inositol (Gaspar, Aregullin et al. 2006) (Figure 34.A). This indicates that the amount

of inositol synthesized *de novo* when the *INO1* gene is fully derepressed is not sufficient to maintain PI levels seen in fully supplemented wild type cells. Consistent with the results of Gaspar et al., 2006, it was observed that wild type cells grown in the absence of inositol exhibited high PC, diacylglycerol (DAG) and triacylglycerol (TAG) levels and low PI and free fatty acid (FFA) levels compared to cells grown in the presence of inositol. The decrease in PC and TAG levels when inositol is present is due to the fact that exogenous inositol affects the utilization of critical shared intermediates in lipid metabolism, such as PA, DAG and CDP-DAG (Figure 1.C). PA, DAG and CDP-DAG levels drop rapidly when inositol is added to growth medium lacking inositol, due to increasing rates of PI synthesis. Increased PI synthesis, in turn, leads to decreased PC synthesis via the PE methylation pathway (Figure 1.C) because less CDP-DAG is available for PS synthesis (Loewen, Gaspar et al. 2004; Gaspar, Aregullin et al. 2006) (Figure 1.C). PI synthesis directly completes with PS synthesis for the CDP-DAG precursor. Therefore, the increased rate of PI synthesis in the presence of inositol results in reduced PS synthesis (Kelley, Bailis et al. 1988) and the lowered rate of PS synthesis subsequently affects PE and PC synthesis via the methylation pathway. The lowered flux of PA to DAG following inositol addition also results in decreased PC synthesis via the CDP-choline pathway. DAG availability as a precursor for TAG synthesis is also decreased in the presence of inositol (Figure 1.C).

Apparently, perhaps due to the changes in membrane lipid synthesis described above, growth in medium lacking inositol is stressful even to wild type cells, since numerous stress response pathways are activated in the absence of inositol. These pathways include the UPR, CWI-PKC, the High Osmolarity Glycerol (HOG) pathways and others (M.Villa-Garcia, S. Jesch and S. Henry, unpublished data) (Cox, Chapman et al. 1997; Chang, Jones et al. 2002; Nunez, Jesch et al. 2008). When choline is added to the growth medium, PC synthesis via the CDP-choline pathway is

highly stimulated, especially when inositol is absent (Gaspar, Aregullin et al. 2006). The presence of choline also results in increased turnover of PC catalyzed by Nte1p, phospholipase B localized to the ER, especially at higher temperatures (Dowd, Bier et al. 2001; Zaccheo, Dinsdale et al. 2004). As discussed previously, the presence of choline and higher growth temperatures also strengthens the Ino$^-$ phenotype of mutants defective in the CWI-PKC pathway, such as *mpk1*Δ (Nunez, Jesch et al. 2008). Furthermore, the Ino$^-$ phenotype of the *mpk1*Δ mutant is suppressed by overexpression of *NTE1*, suggesting that increasing levels of PC contribute to this phenotype (Nunez, Jesch et al. 2008).

It was observed that increasing growth temperature and/or the presence of choline had effects on the Ino$^-$ phenotype of the *npt1*Δ mutant that are similar to the effects they have on the Ino$^-$ phenotype of the *mpk1*Δ mutant. The Ino$^-$ phenotype of the *npt1*Δ mutant is observed only at 37^{o}C and is clearly strengthened by the addition of choline (Figure 3.A and 6). Furthermore, overexpression of *NTE1* partially suppressed the *npt1*Δ Ino$^-$ phenotype, suggesting that elevated PC levels contribute to the *npt1*Δ growth defect (Figure 10.B). Thus, a number of previous reports suggest that both the presence of choline and increased growth temperature influence lipid metabolism in yeast, especially when inositol is absent. Higher growth temperatures and the absence of inositol both trigger UPR signaling (Cox, Chapman et al. 1997; Chang, Jones et al. 2002), in addition to their effect on CWI-PKC signaling (Kamada, Jung et al. 1995; Nunez, Jesch et al. 2008). Furthermore, growth at higher temperatures results in a higher rate of PI synthesis and accumulation, suggesting that cells require more PI under these conditions (Gaspar, Jesch et al. 2008; Ejsing, Sampaio et al. 2009).

When cells were shifted from I+ NA- to I- NA- medium, the changes in phospholipid composition in *npt1*Δ cells were comparable to those in wild type cells

(Figure 35 and 36). However, when the cells were shifted from I+ NA+ to I- NA+ medium, I found that PS and PC levels in the *npt1*Δ strain increased 3-fold and 1.4-fold, respectively, compared with the wild type strain under the identical conditions. Therefore, increased PC levels in *npt1*Δ cells shifted to I- NA+ medium may be contributing to the Ino$^-$ phenotype of the mutant. However, sterol ester (SE) levels in wild type cells grown continuously in medium lacking inositol were higher than levels observed either in *npt1*Δ or wild type cells grown in other growth media. When cells were shifted from I+ to I- medium, whether NA was present or not, SE levels in *npt1*Δ cells were higher than levels in wild type cells under the identical conditions (Figure 37, 38 and 39). Furthermore, 1,2-DAG and TAG levels in *npt1*Δ were significant elevated, in cells shifted to I- NA+ medium, in comparison to wild type cells under all other conditions tested (Figure 37, 38 and 39). Increased TAG levels were also observed in *mpk1*Δ cells shifted to inositol free medium containing choline (Nunez, Jesch et al. 2008) as well as in *sec13-1*, a temperature sensitive mutant defective in membrane trafficking mechanism from the ER. The *sec13-1* mutant also exhibited an Ino$^-$ phenotype and UPR activation at permissive growth temperatures (Gilstring, Melin-Larsson et al. 1999; Chang, Jones et al. 2002; Gaspar, Jesch et al. 2008).

Therefore, I am proposing the hypothesis that the Ino$^-$ phenotype of *npt1*Δ cells may be related to severe stress. Wild type cells grown in the absence of inositol experience stress, activating several stress responses (Jesch, Zhao et al. 2005; Jesch, Liu et al. 2006; Nunez, Jesch et al. 2008). While wild type cells can survive in these conditions, they require the activity of numerous stress response pathways to do so. In mutants lacking a functional UPR, CWI-PKC, or HOG pathway, the addition of inositol rescues their growth under these conditions even at higher growth temperatures (M. Villa-Garcia and S. Henry, unpublished data; (Nunez, Jesch et al. 2008). In future work, I propose to study the activation of stress response in *npt1*Δ and

wild type cells in response to changes in NAD^+ metabolism influenced by inositol and NA availability. Also, it will be wondered whether NAD^+ levels in *npt1*Δ cells following a shift from I+ to I- medium change.

Conclusion : *npt1*Δ cells grow poorly in the absence of inositol at 37°C and this phenotype is made worse by the presence of choline. Thus, the phenotype of *npt1*Δ is similar to that of mutants defective in several stress response pathways. I propose that the presence of NA, which cannot be metabolized by *npt1*Δ, contributes to this stress, since removal of NA from *npt1*Δ growth medium restored both *INO1* expression and phospholipid compositions to levels comparable to wild type under these conditions, when cells were shifted from I+ to I- medium.

C. NAD^+ metabolism is affected by both inositol and NA

C. 1. Change in NAD^+ levels in response to both inositol and NA supplementation

NAD^+ is required for *de novo* inositol synthesis since wild type cells grown in the absence of inositol express Ino1p which requires NAD^+ for its activity (Byun and Jenness 1981; Donahue and Henry 1981). Thus, the need to synthesize inositol *de novo* might place a further demand on NAD^+ biosynthesis, requiring cells to upregulate NAD^+ production. I found that NAD^+ levels in wild type cells grown continuously in I- NA+ medium were 20% higher than the levels in cells grown continuously in I+ NA+ medium (Figure 15). However, wild type cells exhibited increased NAD^+ levels in I- medium only in the presence of NA. In the absence of NA, NAD^+ levels decreased about 50% compared to cells grown in the presence of NA, whether inositol was present or not (Figure 15). Thus, there was no significant difference in the NAD^+ levels seen in cells grown in I+ NA- compared to cells grown continuously in I- NA- medium. Therefore, exogenous NA clearly plays a more

critical role in determining the steady state level of NAD^+ in wild type cells than inositol. However, the fact that the presence of inositol influenced NAD^+ levels, when cells are grown in the absence of NA, indicates that inositol does exert an effect on NAD^+ levels under certain conditions. Interestingly, however, when wild type cells were shifted from I+ NA+ medium to I- NA+ medium, NAD^+ levels actually decreased during the first 3hrs in the new medium (Figure 16). These results suggest that a transient increase in NAD^+ utilization occurs upon a shift to I- NA+ medium before the rate of NAD^+ synthesis is able to adjust. This increased utilization of NAD^+ could be due at least in part to derepression of Ino1p. Wild type cells clearly adjust to this requirement over longer growth periods, since, as discussed above, NAD^+ levels in cells grown continuously in I- NA+ medium actually exceed those of cells fully supplemented with inositol (Figure 16). Therefore, both inositol and NA in the growth medium exert an influence on NAD^+ metabolism and intracellular NAD^+ levels in wild type cells (Figure 15).

To test whether the increase in NAD^+ levels in I- NA+ medium is related to Ino1p activity, I compared NAD^+ levels in the *ino1*Δ strain, which lacks Ino1p, to wild type before and after cells were shifted from I+ NA+ to I- NA+ medium at mid-logarithmic phase. As described above, NAD^+ levels in wild type cells decreased about 2-fold within 3 hrs following the shift to I- NA+ medium, compared to the levels observed in cells grown continuously in I- NA+ medium. In contrast, in the *ino1*Δ mutant within 3 hrs following the shift to I- NA+ medium, NAD^+ levels increased to levels comparable to those in wild type cells grown continuously in I- NA+ medium (Figure 16). Cellular NAD^+ levels in *ino1*Δ cells shifted to I- NA+ medium reached a higher level than those observed in *ino1*Δ or wild type cells grown continuously in I+ NA+ medium (Figure 16). Thus, I propose that *ino1*Δ cells may experience an increased demand for NAD^+, due to changing metabolism produced by stress related

to inositol deprivation, which is known to result in cell death within 4-5hrs following a shift of *ino1*Δ cells to inositol free medium (Henry, Atkinson et al. 1977). I also propose that the transient decline in NAD^+ levels in the wild type strain could be due, at least partially, to increasing Ino1p activity. Clearly, however, the dramatic increase in NAD^+ levels *ino1*Δ cells can not easily be explained by the absence of Ino1p activity. The increased NAD^+ levels in the *ino1*Δ mutant following the shift to I- NA+ medium suggests that a factor or factors other than Ino1p demand for NAD^+ is responsible, at least in part, for the long term rise in NAD^+ levels seen in wild type strain.

In NAD^+ metabolism, Npt1p catalyzes the conversion of NA to NaMN in the salvage pathway (Figure 2.A). In the presence of NA, cells can synthesize NAD^+ efficiently via the salvage pathway, reducing reliance on *de novo* synthesis involving the products of the *BNA* genes (Figure 2.A). However, in the absence of NA, cells must rely on synthesis of NAD^+ from tryptophan via the *de novo* pathway, requiring expression of *BNA* genes. The intermediate, NaMN produced through the *de novo* pathway, is also produced through the action of Npt1p from NA in the salvage NAD^+ pathway. In wild type cells, NAD^+ synthesized by either route is recycled via the salvage pathway (Figure 2.A). However, the *npt1*Δ mutant cannot recycle NAD^+ synthesized by the *de novo* pathway and it cannot use exogenous NA. Consistent with its metabolic block, the *npt1*Δ mutant was reported to have 2-3 fold decreased NAD^+ levels compared to wild type cells grown in YPD or synthetic complete (SC) medium containing NA (Sandmeier, Celic et al. 2002; Bedalov, Hirao et al. 2003). I also observed that the *npt1*Δ strain exhibited consistently low NAD^+ levels under all growth conditions tested, whether cells were grown at 30 or 37^{o}C and whether inositol and/or NA were present or not (i.e., I+ NA+, I- NA+, I+ NA- and I- NA- media) (Figure 17 and 20). Therefore, I hypothesize that low NAD^+ levels and/or the

additional energy required to synthesize every molecule of NAD^+ *de novo* in the *npt1*Δ strain might play a role in the Ino^- phenotype of the mutant. In other words, the constitutively low NAD^+ levels in *npt1*Δ cells may contribute to their inability to adjust to the stress produced by the absence of inositol and higher growth temperatures. Therefore, I propose that the defect in NAD^+ production in *npt1*Δ, which results in constitutively low NAD^+ levels, makes the cell less resilient when confronted with the stress caused by the combination of high temperature and the absence of inositol. Npt1p is essential due to its critical role in maintaining NAD^+ levels through the salvage pathway. It has also been reported that the low NAD^+ levels in the *npt1*Δ strain resulte in decreased Sir2p activity in telomeric and rDNA silencing (Sandmeier, Celic et al. 2002). Furthermore, overexpression of *NPT1* reportedly induced an increase in Sir2p activity resulting in enhanced rDNA silencing, although NAD^+ levels were not found to be changed (Anderson, Bitterman et al. 2002). However, it is important to keep in much that silencing is influenced by nuclear NAD^+ levels and that overall cellular NAD^+ levels may not be an accurate indicaiton of the NAD^+ pool availability to sirtuins localized in the nucleus (Gotta, Strahl-Bolsinger et al. 1997). The salvage pathway is critical to maintain nuclear NAD^+ levels and Npt1p is localized in the nucleus (Anderson, Bitterman et al. 2002; Sandmeier, Celic et al. 2002). In contrast, enzymes involved in the *de novo* pathway are localized to the cytoplasm (Sandmeier, Celic et al. 2002).

In the *pnc1*Δ strain, which accumulates NAM, an inhibitor of sirutins (Landry, Slama et al. 2000; Gallo, Smith et al. 2004), I found that NAD^+ levels were undistinguishable from the wild type strain when cells were grown with NA supplementation with and without inositol. However, NAD^+ levels in the absence of NA in the *pnc1*Δ strain were slightly lower than wild type (Figure 18.A). These results are consistent with the defect in the *pnc1*Δ strain, which in the absence of NA

supplementation is forced to rely on *de novo* synthesis of NAD^+ (Figure 2.A). *pnc1*Δ is incapable of conversion of NAM to NA. However, when exogenous NA is present, *pnc1*Δ can metabolize NAD^+ via the salvage pathway. The low NAD^+ levels in *pnc1*Δ in the absence of NA are also consistent with the weak growth defect observed in the *pnc1*Δ mutant in I- NA- medium at 37^{o}C (Figure 4). Apparently, *pnc1*Δ cells, in which the salvage pathway from NAM is blocked, are able to synthesize sufficient NAD^+ to maintain wild type NAD^+ levels if precursors of NAD^+ (i.e., NA and tryptophan) are present in the growth medium. Consistent with my results, previous reports also showed that NAD^+ levels in *pnc1*Δ were comparable to wild type cells grown in YPD medium (Sandmeier, Celic et al. 2002).

I also observed that the *hst1*Δ mutant had NAD^+ levels undistinguishable from wild type under all conditions tested, i.e., I+ NA+, I- NA+, I+ NA- and I- NA- media (Figure 18.B). However, Bedalov *et al*., 2003 reported that under all growth conditions, the *hst1*Δ strain had consistently high NAD^+ levels regardless of the growth medium (i.e., whether tryptophan and/or NA was present or not). Their results suggested that increased *BNA2* and *TNA1* expression levels resulted high NAD^+ levels regardless of the absence of supplemental precursors of NAD^+. Bedalov *et al*., interpreted this result to be consistent with their hypothesis that Hst1p senses and regulates NAD^+ levels through the repression of these genes. However, in my study, NAD^+ levels in *hst1*Δ cells were reduced in a fashion similar to wild type in the absence of NA (Figure 18.B).

Thus, my results suggest that derepression of the *BNA2* gene in the absence of NA is insufficient, either in wild type or *hst1*Δ to maintain the NAD^+ levels seen in cells exposed to exogenous NA. I cannot explain why the results of Bedalov *et al*., with respect to the NAD^+ levels in the *hst1*Δ mutant, differ from my results. I also found that NAD^+ levels in the *hst1*Δ *npt1*Δ double mutant were low and resembled those in the *npt1*Δ strain under all conditions tested except in I+ NA+ medium (Figure

18.B). Thus, the additional derepression of *de novo* NAD^+ pathway caused by the *hst1*Δ mutation is clearly insufficient to elevate NAD^+ levels in the double mutant carrying the *npt1*Δ mutation, which prevents NAD^+ recycling via the salvage pathway. My result is consistent with my interpretation although the result is in contrast to that of Bedalov *et al*.

Conclusion : NAD^+ levels in wild type cells respond to external environmental conditions including the presence of exogenous inositol and/or NA as well as growth temperature. The low NAD^+ levels observed in the *npt1*Δ strain are insufficient to support increased cellular demand for NAD^+ when inositol is absent especially at high temperature, thus preventing growth under these conditions. I propose that this effect is the major cause of the Ino^- phenotype of the *npt1*Δ strain.

C. 2. Consistent intracellular NA levels in all strains tested

Deletion of *NPT1* results in a block in the conversion of NA to nicotinic acid mononucleotide (NaMN) (Figure 2.A). The presence of exogenous NA may lead to changes in the intracellular environment and/or metabolism in the *npt1*Δ strain that is unlike its effect on wild type or other mutants involved in the NAD^+ metabolism. I found that when NA was omitted from the growth medium, the Ino^- phenotype of the *npt1*Δ strain was partially suppressed (Figure 4) and *INO1* expression levels (Figure 14) and the relative levels of phospholipids and neutral lipids (Figure 36 and 39) in the *npt1*Δ strain were restored to levels comparable to those of the wild type strain grown under comparable conditions. Thus, I hypothesized that *npt1*Δ cells might accumulate more increased intracellular NA in the presence of exogenous NA and that high intracellular NA levels might play a role in the Ino^- phenotype in *npt1*Δ cells. However, intracellular NA levels in all strains tested, *npt1*Δ, *hst1*Δ, and wild type, did not vary greatly in any of the four media tested (i.e., I+ NA+, I- NA+, I+ NA- and I- NA-

media) (Figure 21). These observations suggest that cells have a mechanism for maintaining intracellular NA levels in the face of both changes in NAD^+ metabolism and /or the exogenous supply of NA.

If NA accumulates to levels greater than the amount of cells required, extra NA might be exported out of cells. To investigate this possibility, I tested for the export of NA using the *bna2*Δ strain in a bioassay. The *bna2*Δ strain is an NA auxotroph which relies entirely on salvage pathway from NA for NAD^+ synthesis (Figure 2.A) (Kucharczyk, Zagulski et al. 1998). The *bna2*Δ strain grew on plates lacking NA in the area surrounding all other strains tested, indicating that the wild type, *npt1*Δ and *pnc1*Δ strains all export metabolites capable of supporting *bna2*Δ growth (Figure 22). The *npt1*Δ strain produces NA as a byproduct of metabolism of NAD^+ produced by the *de novo* pathway (Figure 2.A). *npt1*Δ cells may also import large amounts of NA since they express high levels of *TNA1*, encoding the NA transporter, despite the fact that they cannot utilize NA (Figure 32). Therefore, *npt1*Δ cells may have to export a large amount of NA to maintain intracellular NA levels. Moreover, in the case of the *pnc1*Δ strain in the absence of NA, the exported metabolite responsible for the growth of *bna2*Δ, as seen in Figure 22, cannot be NA, since the *pnc1*Δ mutant is blocked in the conversion NAM to NA. Thus, most likely, NAM is the metabolite exported by *pnc1*Δ that is capable of supporting *bna2*Δ growth.

It has been reported that addition of NAM to the growth medium results in increased NAD^+ levels in several mutants involved in NAD^+ metabolism, suggesting that increased NAD^+ levels might be due to the inhibition of Hst1p activity, thus relieving the derepression of the *de novo* pathway (McClure, Gallo et al. 2008). In my study, the *pnc1*Δ mutant exhibited a weak growth defect in I- NA- medium at 37^{o}C (Figure 4), but addition of 5mM NAM suppressed the weak growth defect of the *pnc1*Δ strain in I- NA- medium (Figure 5). Although NAM functions as a metabolite

in the salvage pathway in yeast, extra NAM inhibits the activity of sirtuins through the feedback mechanism. Overall, my results suggest that yeast cells use NA and/or NAM export as a mechanism to regulate cellular NA, NAM and NAD^+ levels. Clearly, since *npt1*Δ cells maintain constant intracellular NA levels, intracellular buildup of NA to toxic levels does not explain the sensitivity of the *npt1*Δ mutant to the presence of NA in I- medium. However, the presence of exogenous NA might cause *npt1*Δ cells to expend extra energy in a futile cycle of importing and exporting NA to a greater degree than other strains.

In wild type cells, the presence of high NA levels results in a reduction in the rate of *de novo* NAD^+ synthesis via the repression of genes involved in *de novo* pathway. However, this does not occur in *npt1*Δ cells, which express high levels of *BNA2* despite the presence of exogenous NA. Thus, it is evident that NA itself is not the metabolite responsible for repression of *BNA2*. Alternatively, the presence of exogenous NA might influence the rate of the conversion from NAM to NA via the reaction catalyzed by Pnc1p, resulting accumulation of NAM and consequentially inhibit the activity of sirtuins (Figure 2.A), although such a feedback mechanism has not bee reported. If *npt1*Δ cells accumulate excess NAM maintaining while intracellular NA levels, the increased NAM levels might result in the inhibition of sirtuin activity. However, this idea remains to be tested

Conclusion : Intracellular NA levels are maintained despite substantial differences in NAD^+ metabolism produced either by mutations or supplementation of the medium with NA or inositol (Figure 21).

C. 3. *TNA1* expression levels are regulated in response to external NA in wild type cells and this regulation requires Hst1p and an intact salvage pathway

The *TNA1* gene encoding the NA transporter is known to be upregulated in the

absence of NA (Klebl, Zillig et al. 2000). The deletion of *HST1* also leads to upregulation of *TNA1* (Bedalov, Hirao et al. 2003). Consistent with the report of Klebl *et al*, 2000, I observed that upregulation of the *TNA1* gene in wild type cells occurs in the absence of NA.

Specifically, when wild type cells were shifted from I+ NA- to I- NA- medium, the *TNA1* gene was expressed at a high level following the shift (Figure 31). In contrast, when wild type cells were shifted from I+ NA+ to I- NA+ medium, they maintained constantly low *TNA1* expression levels following the shift. Thus, I observed no effect on *TNA1* expression levels when cells were shifted to inositol free medium, whether NA was present or not (Figure 31).

In contrast, the cells grown continuously in I+ NA+ or I+ NA- without a shift to fresh medium, as a control, showed subtle changes in *TNA1* expression levels. Specifically, cells grown in I+ NA+ medium showed a gradual increase in *TNA1* expression, while cells grown continuously in I+ NA- medium showed a gradual decline in *TNA1* expression (Figure 21). Cells growing continuously in the same medium are constantly taking up NA or exporting it into the growth medium, thus NA concentration in the medium will be continuously modified. Consistent with this idea, *TNA1* expression levels in wild type cells grown continuously in I+ NA+ and I+ NA- media converged at one intermediate point over the course of the experiment.

Thus, I believe that the convergence of *TNA1* expression levels most likely reflects changing NA concentration in the growth medium due to ongoing NAD^+ synthesis and turnover. In contrast, when the cells were shifted to new media in order to remove the inositol, the new media contained the initial NA concentration, and *TNA1* expression levels remained constant.

Overall, my data clearly confirms that *TNA1* expression in wild type cells responds to NA level in the growth medium. Moreover, the data reveal that the effect

of inositol on *TNA1* expression is likely very small. However, to evaluate the effect of inositol and NA more precisely, it will be necessary to repeat this experiment, shifting the control culture to the fresh medium, (i.e., similar to those cultures in which inositol concentrations were held constant, shifting from I+ NA+ to I+ NA+ or from I- NA- to I- NA- media).

Moreover, I found that *TNA1* expression levels in the *npt1*Δ strain were higher than in wild type at t=0, whether the cells were grown in the presence of NA or not, and were unaffected when cells were shifted to inositol free medium (Figure 32). However, *TNA1* expression levels in the *npt1*Δ strain trended down over the course of the experiment (Figure 32), but did not respond to exogenous NA and inositol.

*hst1*Δ cells shifted to I- NA+ or I- NA- medium, similar to *npt1*Δ cells, exhibited high levels of *TNA1* expression at t=0, whether NA was present or not. However, unlike *npt1*Δ cells, the levels of *TNA1* in *hst1*Δ cells remained high throughout the experiment whether NA was present or not (Figure 33.A). Presumably, this occurs because *hst1*Δ cells cannot sense NAD^+ levels. In contrast, in *npt1*Δ cells, *TNA1* expression is high because NAD^+ is always low (Figure 17 and 32) and thus results in decreased activity of the sirtuin, Hst1p, which acts as a sensor of NAD^+ levels. I did not measure NAD^+ levels throughout this experiment, but it is possible that NAD^+ levels might rise gradually in *npt1*Δ cells as they approach stationary phase. This could explain the gradual decrease in *TNA1* expression. However, my data clearly support the role of Hst1p as a negative regulator of the *TNA1* gene, is necessary for repression of *TNA1* in the presence of NA.

Conclusion : My results generally confirmed the previous report that the *TNA1* gene is regulated in response to exogenous NA levels. The fact that the Hst1p activity is required for this regulation suggests that the cells do not sense NA directly but rather respond to the effect of exogenous NA on NAD^+ levels in wild type cells.

However, in contrast to the increased expression of *BNA2* in response to removal of inositol (Figure 23 and 25), I observed little effect on *TNA1* expression in the absence of inositol. This result suggests that the expression of the *BNA2* and *TNA1* genes differs in sensitivity to the effect of inositol, even though regulation of both genes requires the activity of Hst1p.

C. 4. The pattern of *BNA2* transcription in response to inositol and NA

It has been reported that the *BNA2* gene is upregulated, in a fashion similar to the *TNA1* gene in the absence of NA or when the *HST1* gene is deleted (Bedalov, Hirao et al. 2003). In a previous report involving a microarray study from our laboratory, the *BNA2* gene was found to be repressed several fold following the addition of inositol to medium already containing NA (Jesch, Zhao et al. 2005). In a previous section of Discussion, I have discussed the effect of inositol on NAD^+ levels. Indeed, NAD^+ levels in wild type cells grown continuously in I- NA+ medium were shown to be higher than in any other condition tested (Figure 15). Therefore, I hypothesized that inositol together with NA would result in changes in NAD^+ levels, which in turn would affect *BNA2* expression. Consistent with my hypothesis, *BNA2* expression levels in wild type cells increased when wild type cells were shifted to medium lacking both inositol and NA (I- NA-) (Figure 23). In wild type cells grown continuously in medium lacking inositol with NA, high NAD^+ levels were observed, but when wild type cells were shifted from I+ NA+ to I- NA+ medium, a decrease in NAD^+ levels of 50% was observed within the first 3 hrs following the shift (Figure 16). These results suggest that NAD^+ utilization may increase immediately following the shift to I- medium, but that cells are able to restore NAD^+ homeostasis in the long term when grown continuously in I- medium. Therefore, wild type cells must compensate for the increased NAD^+ demand under these conditions. When NA is present in the

growth medium, wild type cells can adjust NAD^+ production either by importing more exogenous NA or by upregulating *de novo* NAD^+ synthesis. It appears that under these conditions, wild type cells mainly rely on importing NA and use it as a precursor via the salvage pathway to increase NAD^+ production. However, when NA is absent, the only option available for adaptation to increased NAD^+ demand is to rely on increased *de novo* synthesis. Consistent with this reasoning, I observed transient upregulation of *BNA2* expression in wild type cells shifted to I- NA- medium, peaking at 3 hrs (Figure 23). Thereafter, *BNA2* expression levels gradually decreased, suggesting that the cells have acclimated to their new external environment.

However, in contrast to wild type cells, *BNA2* expression in *npt1*Δ cells reached higher levels both when cells were shifted to I- NA+ medium as well as to I- NA- medium at 30°C (Figure 25 and 26). Furthermore, *npt1*Δ cells shifted to I- NA+ medium exhibited continuously increased *BNA2* expression levels, even after 3 hrs, suggesting that continuously elevated expression of *BNA2* in *npt1*Δ cells may be in response to the need to produce higher levels of NAD^+. This observation is consistent with the fact that *npt1*Δ cells must rely on *de novo* synthesis whether NA is present or not. It is also consistent with my hypothesis that *npt1*Δ cells shifted to I- NA+ medium expend more energy when NA is present, because of the need to export NA in order to maintain constant cellular levels of this metabolite. However, at 3hr following the shift to I- NA- medium, *BNA2* expression levels in *npt1*Δ cells were similar to those in wild type cells (Figure 26.B). Under these conditions, *INO1* expression levels were also similar to wild type (Figure 14) as were the levels of phospholipid metabolites (Figure 26.B and 35). Thus, removal of NA from the medium restored both *INO1* and *BNA2* expression in *npt1*Δ cells to levels similar to those seen in wild type.

When the growth temperature was increased to 37°C, *BNA2* levels in wild type cells shifted to I- NA+ medium were 2-fold higher than the levels observed at 30°C

under the same condition. In contrast, the levels of *BNA2* expression in wild type cells shifted to I- NA- were not much different from the levels observed at 30°C (Figure 28 and 30). The levels of *BNA2* expression in wild type cells shifted to I- NA- at both 30 and 37°C were higher than the levels in wild type cells shifted to I- NA+ at 37°C. This result suggests that regardless of temperature, the NAD^+ levels reached when NA is absent reflects the ultimate level, which wild type cells can achieve when relying on the *de novo* pathway. However, *npt1*Δ cells exhibited at least 2-fold higher *BNA2* expression levels at 37°C as compared to 30°C when shifted to I- NA+ or I- NA- (Figure 29 and 30). Furthermore, comparing the results I obtained with the wild type and *npt1*Δ strains, it appears that *BNA2* expression level in wild type responds more to the absence of NA than to higher growth temperatures, whereas *BNA2* expression in *npt1*Δ cells responds more to higher growth temperatures rather than to exogenous NA (Figure 30).

The pattern of *BNA2* expression I observed in wild type cells generally correlated with the observed NAD^+ levels. At 37°C, NAD^+ levels in wild type cells in I+ NA+ medium were higher than the levels at 30°C (Figure 19), suggesting that cells need more NAD^+ to grow at higher growth temperatures and *BNA2* expression is also higher under these conditions. Therefore, I suggest that while *npt1*Δ cells also require more NAD^+ in response to higher growth temperatures, they cannot synthesize sufficient NAD^+ due to the lack of an intact salvage pathway. Indeed, *npt1*Δ cells exhibit consistently low NAD^+ levels even at 37°C (Figure 20). Therefore, the elevated *BNA2* expression in *npt1*Δ cells under these conditions, indicating derepression of the *de novo* pathway, most likely reflects an attempt by cells to produce more NAD^+ to satisfy increased demand, relying completely on *de novo* NAD^+ synthesis.

BNA2 gene repression in response to exogenous NA requires the activity of Hst1p, a NAD^+-dependent protein deacetylase, recruited by Sum1p, a transcriptional

repressor of middle sporulation genes, which also binds to the promoter of genes involved in *de novo* NAD^+ synthesis (Bedalov, Hirao et al. 2003). Sum1p was also reported to be present at the promoters of the *BNA* genes, which are believed to be regulated by Hst1p. However, Sum1p binding to these promoters reportedly does not depend on cellular NAD^+ levels, which suggests that the degree of repression of these genes is modulated by the deacetylase activity of Hst1p (Bedalov, Hirao et al. 2003). Therefore, I expected that deletion of the *HST1* gene would lead to increased *BNA2* expression levels regardless of the presence of inositol and/or NA since Hst1p has been proposed as the sensor of cellular NAD^+ levels. When NAD^+ levels drop, Hst1p is expected to be affected more rapidly than that of other sirtuins because it has the highest Km for NAD^+ concentration drop below the Km for Hst1p, it cannot deacetylate histones in the promoters to which it is bound, leading to their elevated expression (Bedalov, Hirao et al. 2003). This is believed to be the mechanism for derepression of the *BNA* genes in response to lowered NAD^+ levels. However, even though overall *BNA2* expression levels in the *hst1*Δ strain were higher than in either *npt1*Δ or wild type (Figure 29), the transcription of the *BNA2* gene in the *hst1*Δ strain responded dramatically to inositol whether NA was present or not. Following the shift to I- medium whether NA was present or not, *BNA2* expression levels in *hst1*Δ cells were more than 2-fold higher than the highest levels observed in wild type and *npt1*Δ cells shifted to medium lacking inositol (Figure 29). In contrast, wild type cells should a similar transient increased in *BNA2* expression only when NA was absent.

Hst1p clearly plays a critical role in repression of the *BNA2* gene. Low NAD^+ levels in *npt1*Δ cells results in derepression of *BNA2* gene, especially when cells are shifted to medium lacking inositol. Deletion of *HST1* stimulated an even more dramatic increase in *BNA2* expression levels than those observed in *npt1*Δ cells. This result is consistent with role of Hst1p as the negative regulator of the *BNA2* gene.

However, my results clearly revealed that the mechanism controlling the transient high level of expression of *BNA2* in response to a shift medium lacking inositol is not dependent on Hst1p since the transient increase in expression occurs even in *hst1*Δ cells shifted to medium lacking inositol. Thus, there might be another factor regulating *BNA2* gene. In the study of Bedalov *et al*. (2003), the upregulation level of *BNA* genes in the *sum1*Δ strain was higher than in the *hst1*Δ strain in microarray data. It is possible that an additional transcription factor or another sirtuin in addition to Hst1p may bind to Sum1p to regulate *BNA2* transcription.

Conclusion : Overall my data clearly support the interpretation that the pattern of *BNA2* expression responds to the overall cellular demand for NAD^+ through the activity of Hst1p and that NAD^+ demand is influenced by the availability of inositol and growth temperature as well as NA.

D. Conclusion

The data presented in this thesis demonstrates that lack of inositol supplementation influences NAD^+ metabolism in yeast. The data I reported indicates that immediately after a shift to medium lacking inositol, cells adjust both NAD^+ and lipid metaoblism. Rapid depletion of inositol appears to result in increased NAD^+ demand and this demand is not simply due to the NAD^+ requirement of Ino1p, the enzyme that catalyzes the rate limiting step in the *de novo* synthesis of inositol (Figure 16). Immediately after a shift to inositol free (I-) medium, especially in the absence of NA, wild type cells exhibit increased expression of *BNA2*, a gene encoding a key enzyme in the pathway for the *de novo* synthesis of NAD^+. Since *BNA2* expression is known to be regulated in response to cellular NAD^+ levels, this increased expression level is presumably due to transient NAD^+ demand.

*npt1*Δ cells are able to respond to such increase NAD^+ demand only by

derepression of the genes, such as *BNA2* because they lack the NAD^+ salvage pathway. Under most metabolic situations, *npt1*Δ cells can satisfy their NAD^+ needs by derepression of the *de novo* pathway for synthesis of NAD^+. However, under stressful conditions, such as those caused by the lack of inositol and higher growth temperatures, higher NAD^+ demand in the *npt1*Δ strain may lead to an overall decrease in the activity of sirutins, such as Sir2p, due to low NAD^+ levels. Additional studies will be necessary to test this hypothesis by assaying Sir2p modulated silencing and *BNA2* expression correlated to NAD^+ levels in wild type, *npt1*Δ and *hst1*Δ cells shifted to I- medium in the presence and absence of NA .

Consistently low NAD^+ levels or the addition of NAM is known to result in decreases in the activity of sirtuins. It is reported that under stress conditions, such as calorie restriction, osmotic stress and heat stress, the expression of sirtuins is increased (Kaeberlein, Andalis et al. 2002; Anderson, Bitterman et al. 2003). Moreover, overexpression of sirtuin genes can protect against such stress. For example, strains carrying additional copies of *SIR2* were significantly resistant to heat shock stress compared with the wild type strain (Anderson, Bitterman et al. 2002). However, I did not observe any suppression of the *npt1*Δ Ino^- phenotype by overexpression of *SIR2* or *HST1*. However, these experiments were inconclusive due to the Ino^- phenotype conferred by the overexpression in *npt1*Δ of the 2μ plasmid I employed (Figure 9.A and B.). However, Hst1p appears to play a role in the Ino^- phenotype of *npt1*Δ (Figure 4). It will be necessary to analyze the role of other sirtuins to determine how their activities affect NAD^+ metabolism in the presence and absence of inositol using double mutants, i.e., *hst1*Δ *sir2*Δ, *hst1*Δ *hst2*Δ, or *hst1*Δ *hst3*Δ mutants or other single sirtuin mutants, i.e., *hst3*Δ and *hst4*Δ mutants under the conditions that lead to the Ino^- phenotype of the *npt1*Δ strain.

Furthermore, it will be interesting to elucidate the effect of sirutins, in

contribution with inositol and NA deprivation, on stress response signaling, such as the UPR, CWI-PKC and HOG pathways, by using microarry analysis.

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